

FORM PTO-1390
(REV 10-94)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

270.60USWO

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

Unknown 09/937452 ✓

INTERNATIONAL APPLICATION NO.

PCT/CA00/00305 ✓

INTERNATIONAL FILING DATE

March 24, 2000 ✓

PRIORITY DATE CLAIMED

March 25, 1999 ✓

TITLE OF INVENTION

MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL PLANTS

APPLICANT(S) FOR DO/EO/US

SAXENA et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
- ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
- ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
- ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Article 34 Amendment; Front page of published PCT application; Notification of Receipt of Demand; PCT/IB/332; PCT Request; PCT/IB/304; International Preliminary Examination Report with amended pages; International Search Report; Written Opinion

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unknown 09/937452		INTERNATIONAL APPLICATION NO PCT/CA00/00305		ATTORNEY'S DOCKET NUMBER 270.60USWO	
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17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a) (1)-(5)): Search Report has been prepared by the EPO or JPO.....\$860.00 International preliminary examination fee paid to USPTO (37 CFR 1.492(a)(1)).....\$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....\$710.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(3)) paid to USPTO \$1000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)\$100.00				CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	59	-20 = 39	X \$18.00	\$702.00	
Independent claims	3	-3 = 0	X \$80.00	\$0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$270.00	
TOTAL OF ABOVE CALCULATIONS =				\$1832.00	
Reduction by 1/2 for filing by small entity, if applicable. Small entity status is claimed pursuant to 37 CFR 1.27				\$0	
SUBTOTAL =				\$1832.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+ \$0	
TOTAL NATIONAL FEE =				\$1832.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				+ \$0	
TOTAL FEES ENCLOSED =				\$1832.00	
				Amount to be: refunded	\$0
				charged	\$0

a. ☐ Check in the amount of \$_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 13-2725 in the amount of \$ 1,832.00 to cover the above fees.
 A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
 overpayment to Deposit Account No. 13-2725.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO
 Gregory A. Sebald
 MERCHANT & GOULD
 P.O. Box 2903
 Minneapolis, MN 55402-0903

SIGNATURE:

NAME: Gregory A. Sebald

REGISTRATION NUMBER: 33,280

S/N unknown

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	SAXENA et al.	Docket No.:	270.60USWO
Serial No.:	unknown	Filed:	concurrent herewith
Int'l Appln No.:	PCT/CA00/00305	Int'l Filing Date:	March 24, 2000
Title:	MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL PLANTS		

CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number: EL669941883US

Date of Deposit: September 25, 2001

I hereby certify that this correspondence is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

By:

Name: Omesh Singh

PRELIMINARY AMENDMENT

Box PCT

Assistant Commissioner for Patents

Washington, D.C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendment, which is based on the Article 34 amendments, based on claims amended in prosecution of the international application and published in the International Preliminary Examination Report, a copy of which is enclosed herewith:

IN THE ABSTRACT

Insert the attached Abstract page into the application as the last page thereof.

IN THE SPECIFICATION

A courtesy copy of the present specification is enclosed herewith. However, the World Intellectual Property Office (WIPO) copy should be relied upon if it is already in the U.S. Patent Office.

IN THE CLAIMS

Please amend the following claims:

5. (Amended) The method according to claim 1, wherein said
phytopharmaceutical plant is selected from the group consisting of:

Achillea millefolium

Achyranthes bidentata

Aconitum napellus

Adonis aestivalis

Agastache mexicana

Agrimonia eupatoria

Agathosma betulina

Allium sp

Anchusa officinalis

Anemopsis californica

Angelica dahurica

Angelica polymorpha sinensis (*A. sinensis*)

Arnica Montana

Ammi visnaga

Arctostaphylos uva-ursi

Asclepias tuberosa

Astragalus membranaceus

Astragalus chinensis

Baphicacanthus cusia

Bixa orellana
Bupleurum falcatum
Brugmansia (Datura) spp.
Campanula rapunculus
Carum roxburgianum
Carum copticum
Cassia tora
Chamaelirium luteum
Chimaphila umbellata
Commiphora africana
Conium maculatum
Crithium maritimum
Datura metel (Datura alba)
Datura inoxia
Dracocephalum moldavica
Echinacea sp.
Eclipta alba (E. prostrata)
Ephedra nevadensis
Eriodictyon californicum
Eucommia ulmoides
Eupatorium perfoliatum
Filipendula vulgaris (F. hexapetala)
Gaultheria procumbens

Geum urbanum
Houttuynia cordata
Hydrocotyle asiatica (Centella asiatica)
Hypericum perforatum cv. Anthos
Inula helenium
Jatropha curcas
Leptospermum scoparium
Lespedeza capitata
Ligusticum porteri
Ligustrum lucidum
Lithospermum officinale
Lycium barbarum
Mucuna pruriens
Mandragora officinarum
Origanum dictamnus
Parietaria judaica (P. officinalis)
Phyllanthus emblica
Picrasma excelsa
Piniella ternate
Pogostemon patchouli
Polygonum multiflorum
Porophyllum ruderale ssp. macrocephalum
Prunella vulgaris

Pueraria lobata (P. thunbergiana)
Rauvolfia serpentina
Rivea corymbosa
Sanguinaria Canadensis
Satureja douglasii
Schizonepeta tenuifolia
Scutellaria baicalensis
Solanum xanthocarpum (S. surattense)
Sutherlandia frutescens
Tabebuia impetiginosa
Tribulus terrestris
Trichosanthes kirilowii
Turnera diffusa
Voacanga africana, and
Withania somnifera

7. (Amended) The method according to claim 1, wherein said at one plant growth regulator having cytokinin activity is selected from the group consisting of thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (*N*-(2-chloro-4pyridyl)-*N'*-(phenyl urea) and 2-*I*-P (*N*6-(2-isopentenyl) adenine or 6-gamma, gamma-dimethylallylamino purine).

11. (Amended) The method according to claim 1, wherein said explant is selected from the seed, petiole, hypocotyl, stem, cotyledon and leaf.

12. (Amended) The method according to claim 1, wherein said phytopharmaceutical plant is St. John's wort.

19. (Amended) The method according to claim 1, wherein the phytopharmaceutical plant is *Echinacea sp.*.

26. (Amended) The method according to claim 1, where said phytopharmaceutical plant is Huang qin.

33. (Amended) The method according to claim 1, wherein the phytopharmaceutical plant is feverfew.

43. (Amended) The method according to claim 2, wherein, in said transferring step, said regenerated tissue is subcultured for about 1 to about 15 days.

44. (Amended) A method for phytofortification of an *in vitro*-grown phytopharmaceutical plant comprising:

a) culturing a sterile seedling, explant or regenerated tissues to form a plantlet;

and

b) subculturing said plantlet onto a basal medium containing at least one additive of interest, to allow uptake and accumulation of said at least one additive of interest in a bio-available form in said plantlet.

45. (Amended) The method according to claim 44, wherein, in said step of culturing, said plantlets are produced either:

- a) on a sterile explant of said phytopharmaceutical plant grown on an induction medium comprising at least one plant growth regulator having cytokinin activity, or
- b) grown from a sterile seed, or
- c) seedling in culture.

46. (Amended) The method according to claim 45, wherein said at one plant growth regulator having cytokinin activity is selected from the group consisting of thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N(-phenyl urea) and 2-*I*-P (N6-(2-isopentenyl) adenine or 6-gamma, gamma-dimethylallylamino purine).

47. (Amended) A phytopharmaceutical plant prepared by the method of claim 1 and comprising an elevated level of said additive of interest when compared to a plant grown in the absence of said additive of interest.

48. (Amended) A method for the *in vitro* micropropagation involving *de novo* shoot formation of non-meristematic tissue of a phytopharmaceutical plant comprising:

a) culturing a steril explant of said phytopharmaceutical plant on an induction medium comprising one or more plant growth regulators having cytokinin activity, to form regenerated tissue; and

b) transferring said regenerated tissue to a basal medium and culturing to form plantlets.

REMARKS

The above preliminary amendment is made to make minor editorial corrections and to remove multiple dependencies from claims 5, 7, 11, 12, 19, 26, 33 and 47.

A new abstract page is supplied to conform to that appearing on the publication page of the WIPO application, but the new Abstract is typed on a separate page as required by U.S. practice.

Applicants respectfully request that the preliminary amendment described herein be entered into the record prior to calculation of the filing fee and prior to examination and consideration of the above-identified application.

If a telephone conference would be helpful in resolving any issues concerning this communication, please contact Applicants' primary attorney-of record, Gregory A. Sebald (Reg. No. 33,280), at (612) 336.4728.

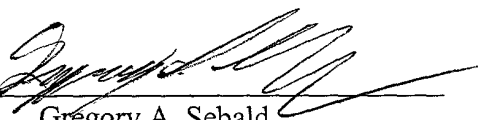
Respectfully submitted,

MERCHANT & GOULD P.C.
P.O. Box 2903
Minneapolis, Minnesota 55402-0903
(612) 332-5300

Dated: September 25, 2001

GAS/tvm

By



Gregory A. Sebald
Reg. No. 33,280

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5. (Amended) The method [of any one of claims 1 to 4] according to claim 1, wherein said
phytopharmaceutical plant is selected from the group consisting of:

Achillea millefolium

Achyranthes bidentata

Aconitum napellus

Adonis aestivalis

Agastache mexicana

Agrimonia eupatoria

Agathosma betulina

Allium sp

Anchusa officinalis

Anemopsis californica

Angelica dahurica

Angelica polymorpha sinensis (A. sinensis)

Arnica Montana

Ammi visnaga

Arctostaphylos uva-ursi

Asclepias tuberosa

Astragalus membranaceus

Astragalus chinensis

Baphicacanthus cusia

Bixa orellana

Bupleurum falcatum

Brugmansia (Datura) spp.

Campanula rapunculus

Carum roxburgianum

Carum copticum

Cassia tora

Chamaelirium luteum

Chimaphila umbellata

Commiphora africana

Conium maculatum

Crithium maritimum

Datura metel (Datura alba)

Datura inoxia

Dracocephalum moldavica

Echinacea sp.

Eclipta alba (E. prostrata)

Ephedra nevadensis

Eriodictyon californicum

Eucommia ulmoides

Eupatorium perfoliatum

Filipendula vulgaris (F. hexapetala)

Gaultheria procumbens

Geum urbanum

Houttuynia cordata

Hydrocotyle asiatica (Centella asiatica)

Hypericum perforatum cv. Anthos

Inula helenium

Jatropha curcas

Leptospermum scoparium

Lespedeza capitata

Ligusticum porteri

Ligustrum lucidum

Lithospermum officinale

Lycium barbarum

Mucuna pruriens

Mandragora officinarum

Origanum dictamnus

Parietaria judaica (P. officinalis)

Phyllanthus emblica

Picrasma excelsa

Piniella ternate

Pogostemon patchouli

Polygonum multiflorum

Porophyllum ruderale ssp. macrocephalum

Prunella vulgaris

Pueraria lobata (P. thunbergiana)

Rauvolfia serpentina

Rivea corymbosa

Sanguinaria Canadensis

Satureja douglasii

Schizonepeta tenuifolia

Scutellaria baicalensis

Solanum xanthocarpum (S. surattense)

Sutherlandia frutescens

Tabebuia impetiginosa

Tribulus terrestris

Trichosanthes kirilowii

Turnera diffusa

Voacanga africana, and

Withania somnifera

7. (Amended) The method according to [any one of claims 1 to 6] claim 1, wherein said at one plant growth regulator having cytokinin activity is selected from the group consisting of thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N(-phenyl urea) and 2-*I*-P (N6-(2-isopentenyl) adenine or 6-gamma, gamma-dimethylallylamino purine).

11. (Amended) The method according to [any one of claims 1 to 10] claim 1, wherein said explant is selected from the seed, petiole, hypocotyl, stem, cotyledon and leaf.

12. (Amended) The method according to [any one of claims 1 to 10] claim 1, wherein said phytopharmaceutical plant is St. John's wort.

19. (Amended) The method according to [any one of claims 1 to 4] claim 1, wherein the phytopharmaceutical plant is *Echinacea sp.*.

[24] 26. (Amended) The method according to [any one of claims 1 to 4] claim 1, where said phytopharmaceutical plant is Huang qin.

33. (Amended) The method according to [any one of claims 1 to 4] claim 1, wherein the phytopharmaceutical plant is feverfew.

[44] 43. (Amended) The method according to claim 2, wherein, in said transferring step, said regenerated tissue is subcultured for about 1 to about 15 days.

[45] 44. (Amended) A method for phytofortification of an *in vitro*-grown phytopharmaceutical plant comprising:

- a) culturing a sterile seedling, explant or regenerated tissues to form a plantlet; and
- b) subculturing said plantlet onto a basal medium containing at least one additive of interest, to allow uptake and accumulation of said at least one additive of interest in a bio-available form in said plantlet.

[46] 45. (Amended) The method according to claim [45] 44, wherein, in said step of culturing, said plantlets are produced either:

- a) on a sterile explant of said phytopharmaceutical plant grown on an induction medium comprising at least one plant growth regulator having cytokinin activity, or
- b) grown from a sterile seed, or
- c) seedling in culture.

[47] 46. (Amended) The method according to claim [46] 45, wherein said at one plant growth regulator having cytokinin activity is selected from the group consisting of thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N(-phenyl urea) and 2-*I*-P (N6-(2-isopentenyl) adenine or 6-gamma, gamma-dimethylallylamino purine).

[48] 47. (Amended) A phytopharmaceutical plant prepared by the method of [any one claims 1 to 4, or 45 to 47] claim 1 and comprising an elevated level of said additive of interest when compared to a plant grown in the absence of said additive of interest.

[49] 48. (Amended) A method for the *in vitro* micropropagation involving *de novo* shoot formation of non-meristematic tissue of a phytopharmaceutical plant comprising:

- a) culturing a steril explant of said phytopharmaceutical plant on an induction medium comprising one or more plant growth regulators having cytokinin activity, to form regenerated tissue; and
- b) transferring said regenerated tissue to a basal medium and culturing to form plantlets.

12/pst

MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL PLANTS

Field of the Invention

The present invention relates to a method for the micropropagation of phytopharmaceutical plants. Furthermore, the present invention relates to a method for the fortification of phytopharmaceutical plants with nutrients, minerals or other compounds, and to plants obtained using this method.

Background of the Invention

Medicinal plants play an important role in health care throughout the world -especially in non-industrialized continents such as Africa, South America and parts of Asia. Even in many industrialized countries, a number of traditional plants are widely used by a majority of people for minor to moderate everyday ailments through self-medication.

Although many traditional medicinal plant remedies do not undergo extensive scientific testing, they are very popular and their sale is not restricted by government regulatory agencies. Some medicinal plants do have substantial laboratory and clinical testing and those that fall into this category are referred to as phytopharmaceuticals.

One of the major problems associated with phytopharmaceutical plant preparations is the variability in the content of the medicinally active ingredients. This problem was highlighted in Belgium in 1997, when more than 100 people were diagnosed with total destruction of their kidneys through irreversible interstitial fibrosis caused by a mis-identified Chinese medicinal plant (Betz, 1998). This has led to strict government controls on purity and levels of active constituents in phytopharmaceutical products in Europe. Such strict regulation does not currently exist, however, in most countries, including Canada and the United States.

The variability in medicinal content of phytopharmaceutical plants is likely the result of a variety of factors including: year-to-year and plant-to-plant variability in medicinal content; adulteration of medicinal preparations with misidentified plant species; a lack of adequate methods for production and standardization of the crop; a lack of understanding of the unique plant physiology or efficacy with human consumption; and consumer fraud. In addition, phytopharmaceutical plant preparations are typically produced from field-grown crops and therefore are susceptible to infestation by bacteria, fungi and insects that can alter the medicinal content of the preparations.

Past and current efforts have centered on ensuring that preparations of phytopharmaceutical plants contain the correct plant material, that the plant material is processed according to a standardized protocol and that the finished product contains specific levels of a specific marker compound. Another approach has been the application of traditional pharmaceutical development methods to isolate a single "active" component and to synthesize versions of the so-called "drug". This process usually involves the dissection of the plant into chemical components and attempts to identify a single compound responsible for the induction of the desired effects in mammals. Most phytopharmaceutical plant preparations are formulated from whole plants and contain a variety of compounds which may be working synergistically to produce the desired effect. Although Drug Identification Numbers (DIN) have been issued for various preparations on the basis of a standard concentration of a marker compound, there may be no physiological effect in humans which can be directly attributed to this marker compound. The lack of any real knowledge base for the phytopharmaceutical industry has led to the current situation in which the sale of phytopharmaceutical plant preparations is driven largely by enthusiasm rather than solid scientific research. Therefore, with the currently available methods, there is no way to ensure quality, efficacy or safety of phytopharmaceutical plant preparations.

Deficiency of trace minerals in humans may occur as the result of inadequate intake of the mineral in the diet or decreased or impaired absorption in the presence of adequate dietary intake (Whittaker, 1998). The problem of mineral deficiency is most prevalent in developing countries where there may be a dietary imbalance in the nutrient composition. It is estimated that more than 2 billion people worldwide suffer from mineral deficiencies, exhibited as common ailments and diseases (for example, zinc deficiency may lead to low fertility; iodine deficiency may be expressed as muscle and thyroid-related ailments; vitamin C deficiencies render susceptibility to common viruses and colds). Factors that decrease or impair the absorption of minerals and nutrients include dietary constituents which as phytate or fiber, drugs or other chemicals that can interact with essential trace minerals and interactions between essential nutrients (Whittaker, 1998).

The conventional approaches to overcome these deficiency-related ailments are dietary supplements (e.g. vitamin C tablets, zinc lozenges), and consumption of fortified food (iron fortified baby foods; vitamin D fortified milk; iodized salt). Foods commonly fortified with mineral nutrients include flour, bakery goods, rice, macaroni products, breakfast cereals and infant formula (Whittaker, 1998). The efficacy of fortification of foods with inorganic mineral compounds, for example $\text{Zn}(\text{SO}_4)_2$, is limited by the low bioavailability of the ions and the high degree of loss through excretion. Recent studies on the bioavailability of fortified nutrients and vitamins in humans have indicated that greater than 2/3 of the nutrients and additives may not be absorbed by the human gastrointestinal system, as they are not in a bioavailable form. In 1993, the Consultant Group on International Agricultural Research (CGIAR) suggested that increasing the mineral uptake of plants could be used to address the problems associated with deficiency of zinc and other nutrients (Ruel and Bouis, 1998).

Echinacea products are currently among the best-selling herbal remedies in North America and have been for several years (Schardt, 1998). Preparations of

Echinacea sp. have historically been used for the treatment of common human ailments such as colds and flu (Kindscher, 1992). Commercially prepared extracts and whole dried tissue preparations are made from the root of *Echinacea* species, a crop which takes about 3 years to produce a saleable product.

Commercial preparations of *Echinacea* are frequently supplemented with inorganic zinc to increase medicinal efficacy. Zinc supplements are recommended for the maintenance of good health, enhanced immune system function, reduced expression of viral symptoms, tissue formation and for the metabolism of proteins, fats and carbohydrates (Gibson et al., 1998). In commercial preparations, zinc is added to the *Echinacea* root material during processing in the inorganic form $\text{Zn}(\text{SO}_4)_2$. Therefore the absorbance of the supplemented Zn is limited by the low bioavailability. Researchers have reported that certain amino acids, cysteine-containing peptides and organic acids, released during digestion may enhance zinc absorption, possibly by forming soluble ligands with zinc or by preventing the formation of the insoluble zinc-phytate complex (Gibson et al., 1998).

Zinc deficiency is one of the leading causes of limited growth rate, loss of appetite, skin lesions, delayed wound healing, hypogonadism, delayed sexual maturation and impaired immune responses in mammals (Whittaker, 1998).

The process of controlling zinc status is a tightly regulated balance of absorption and excretion processes. Zinc is more efficiently absorbed in small amounts than at higher concentrations and other dietary factors such as fiber and phytate inhibit zinc absorption. In addition, the practice of fortifying foods with iron may have a negative impact on zinc status (Whittaker, 1998). Therefore, the recommendations of the American Food and Drug Administration encourage the prudent use of nutrients as supplements to foods but does not encourage the fortification of food staples.

Similarly, zinc fortification of animal feeds is monitored under the guidelines of Agriculture and Agri-Food Canada and limited to less than 250 ppm. In the USA, ongoing research has indicated that supplementation of animal feeds with 3000 ppm inorganic zinc results in more rapid muscle and protein accretion and an overall acceleration in the rate of growth.

One of the most popular medicinal plants in North America is St. John's wort (*Hypericum perforatum*). In 1998, 7.5 million Americans used St. John's wort for the treatment of neurological disorders and depression (Greenwald, 1998) based on a demonstrated efficacy in numerous clinical trials (Linde et al., 1996). In 1997, the National Institute of Health, Office of Alternative Medicine invested \$4.3 million dollars in a 3-year clinical trial to compare the effects of St. John's wort, a placebo and a standard anti-depressive drug in patients suffering from mild depression (NIH, 1997). The standard for St. John's wort preparations is "the whole fresh or dried plant or its components, including not less than 0.04% naphthodianthrone of the hypericin group calculated as hypericin." (St. John's wort Monograph, 1997) but a recent clinical trial demonstrated that the therapeutic effect of St. John's wort was correlated to the concentration of a second compound hyperforin, (Laakmann et al., 1998). Similar to synthetic antidepressants, the activity of hyperforin was found to cause inhibition of uptake of serotonin, dopamine, noradrenaline, GABA and L-glutamate in animal cell cultures (Chatterjee et al., 1998). Further confounding the situation, studies into the unique physiology of St. John's wort have identified more than 25 additional compounds that may have medicinal activity (Miller, 1998; Nahrstedt and Butterweck, 1997; Evans and Morgenstern, 1997) including the recent report of the presence of relatively high levels of the mammalian neurohormone melatonin (Murch et al., 1997). Therefore, it is clear that the neurological efficacy requires whole plant preparations of St. John's wort.

Lithium, is a mineral element also used in the treatment of depression and

neurological disorders. Frequently, traditional pharmaceutical antidepressant therapies can be augmented with lithium in those situations where a patient's depression is either treatment-resistant or partially and/or insufficiently responsive to treatment (Schweitzer & Tuckwell, 1998). Lithium treatment has been used for more than 40 years and has been found to be effective in 60-80% of all patients although the mode of action is not clear. Toxicity of lithium salts can occur when the blood lithium becomes elevated as a result of fever, diabetes, weight loss diets, salt restricted diets or diarrhea.

It is important to solve the problem of the variability in the medicinal content of preparations of phytopharmaceutical plants so that they can be used reliably and effectively. One solution to producing a reliable source of phytopharmaceutical plants is the development and application of *in vitro* micropropagation procedures to these plants. However, to date, there is no known general method for the *in vitro* micropropagation of phytopharmaceutical plants.

Furthermore, the phytofortification of phytopharmaceutical plants is desired in order to produce plants that comprise desired additives, nutrients and other compounds of interest that are available in a biocompatible form.

There is, therefore, a need within the phytopharmaceutical plant industry for the development of an *in vitro* system for the reliable and reproducible propagation of phytopharmaceutical plants, and when desired the phytofortification of phytopharmaceutical plants with desired compounds of interest..

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

Summary of the Invention:

The present invention relates to a method for the micropropagation of phytopharmaceutical plants. Furthermore, the present invention relates to a method for the fortification of phytopharmaceutical plants with nutrients, minerals or other compounds, and to plants produced using this method.

The present invention provides a method (A) for the *in vitro* micropropagation of a phytopharmaceutical plant comprising:

a) culturing a sterile explant of the phytopharmaceutical plant on an induction medium comprising at least one plant growth regulator having cytokinin activity, to form regenerated tissue; and

b) transferring the regenerated tissue to a basal medium and culturing to form plantlets.

This invention is also directed to the method (A) as defined above wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea* sp., feverfew (*Tanacetum parthenium*) and garlic (*Allium* sp.).

The invention is relates to the method (A) as defined above wherein the plant growth regulator having cytokinin activity may be natural or synthetic and is selected from the group consisting of thidiazuron (TDZ; *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N(-phenyl urea), and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma,gamma-dimethylallylamino purine).

In another of its aspects, the present invention also includes a method (B) for

the *in vitro* micropropagation of a phytopharmaceutical plant comprising:

- a) culturing a sterile explant of the phytopharmaceutical plant on an induction medium comprising a suitable concentration of at least one plant growth regulator having cytokinin activity, to form regenerated tissue;
- b) transferring the regenerated tissue to a basal medium and subculturing to optimize formation of regenerated tissue; and
- c) transferring the regenerated tissue to a basal medium and culturing to form plantlets.

This invention is also directed to the method (B) as defined above wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea* sp., feverfew (*Tanacetum parthenium*) and garlic (*Allium* sp.).

The present invention is also directed to the method (B) as defined above wherein the plant growth regulator having cytokinin activity may be natural or synthetic and is selected from the group consisting of thidiazuron (TDZ; *N*-phenyl-*N'*-(1,2,3-thidiazol-yl) urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N-(phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma,gamma-dimethylallylamino purine).

In another aspect, the present invention provides a method (C) for fortification of an *in vitro* grown phytopharmaceutical plant comprising:

- a) culturing a sterile seedling, explant or regenerated tissue to form a plantlet; and
- b) subculturing the plantlet onto a basal medium containing at least one additive of interest and allowing uptake and accumulation of the at least one additive of interest in a bioavailable form within the plantlet.

Preferably, the sterile explant is cultured in step (a) of Method (C) on a medium comprising at least one growth regulator having cytokinin activity under conditions suitable to form regenerated tissue.

In another aspect, the present invention also provides a method (D) for the *in vivo* fortification of a phytopharmaceutical plant comprising:

a) culturing a plantlet or seedling using method (A) as described above, under conditions sufficient for clonal micropropagation and growth of the plantlet or seedling; and

b) adapting the plantlet or seedling to a hydroponic environment with a recycling solution containing at least one additive of interest to allow uptake and accumulation of the at least one additive of interest in a bio-available form within plantlet.

The phytopharmaceutical plant used in any one of methods (A), (B), (C) and (D) may be selected from:

Achillea millefolium
Achyranthes bidentata
Aconitum napellus
Adonis aestivalis
Agastache mexicana
Agrimonia eupatoria
Agathosma betulina
Allium sp
Anchusa officinalis
Anemopsis californica
Angelica dahurica
Angelica polymorpha sinensis (A. sinensis)
Arnica Montana

Ammi visnaga
Arctostaphylos uva-ursi
Asclepias tuberosa
Astragalus membranaceus
Astragalus chinensis
Baphicacanthus cusia
Bixa orellana
Bupleurum falcatum
Brugmansia (Datura) spp.
Campanula rapunculus
Carum roxburgianum
Carum copticum
Cassia tora
Chamaelirium luteum
Chimaphila umbellata
Commiphora africana
Conium maculatum
Crithium maritimum
Datura metel (Datura alba)
Datura inoxia
Dracocephalum moldavica
Echinacea sp.
Eclipta alba (E. prostrata)
Ephedra nevadensis
Eriodictyon californicum
Eucommia ulmoides
Eupatorium perfoliatum
Filipendula vulgaris (F. hexapetala)
Gaultheria procumbens

Geum urbanum
Houttuynia cordata
Hydrocotyle asiatica (Centella asiatica)
Hypericum perforatum cv. Anthos
Inula helenium
Jatropha curcas
Leptospermum scoparium
Lespedeza capitata
Ligusticum porteri
Ligustrum lucidum
Lithospermum officinale
Lycium barbarum
Mucuna pruriens
Mandragora officinarum
Origanum dictamnus
Parietaria judaica (P. officinalis)
Phyllanthus emblica
Picrasma excelsa
Piniella ternate
Pogostemon patchouli
Polygonum multiflorum
Porophyllum ruderale ssp. macrocephalum
Prunella vulgaris
Pueraria lobata (P. thunbergiana)
Rauvolfia serpentina
Rivea corymbosa
Sanguinaria Canadensis
Satureja douglasii
Schizonepeta tenuifolia

Scutellaria baicalensis
Solanum xanthocarpum (S. surattense)
Sutherlandia frutescens
Tabebuia impetiginosa
Tanacetum parthenium
Tribulus terrestris
Trichosanthes kirilowii
Turnera diffusa
Voacanga africana
Withania somnifera

The nutrient used in method (C) or (D) may be selected from, but not limited to, boron, calcium, chloride, chromium, cobalt, copper, iron, lithium, iodine, magnesium, manganese, molybdenum, nickel, phosphorous, potassium, selenium, silicon, sodium, sulphur, tin, vanadium and zinc.

The production of phytopharmaceutical plants *in vitro* has several advantages:

- plants are grown in sterile, standardized conditions;
- individual superior plants can be identified and clonally produced;
- plant material is consistent and therefore, precise biochemical characterizations can be achieved; and
- eventually, protocols can be developed for the improvement of the crop through genetic manipulation.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the described features.

These and other aspects of the present invention will be described in greater

detail hereinbelow.

Brief Description of the Drawings:

The features of the present invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

Figure 1 shows a sketch of a bioreactor system suitable for use with the methods of the invention.

Figure 2 shows St. John's wort as produced according to the present invention. Figure 2(a) shows TDZ-induced regenerants on etiolated hypocotyl explants of St. John's wort with $10 \mu\text{mol} \cdot \text{L}^{-1}$ TDZ. Pigmentation at the tip of St. John's wort shoot primordia, caused by the presence of the compound hypericin, is visible. (bar = 1 mm). Figure 2(b) shows the development of regenerants on hypocotyl explants after 9 days of culture on TDZ-containing induction medium and 9 days of subculture on basal medium. *De novo* shoots developed from all areas of the explant without an intermediate callus phase in St. John's wort. (bar = 0.75 cm). Figure 2(c) shows sterile cultured plantlets grown from a single hypocotyl section after 2 months on basal medium in a Magenta box. (bar = 1.2 cm)

Figure 3 shows St John's wort as produced according to the present invention. Figure 3 (a) is a histological characterization showing *de novo* shoot induction of St. John's wort and meristematic zones formed at the hypodermal region of the hypocotyl explant (arrows) at 7 days of culture on TDZ-containing induction medium. (bar = $50 \mu\text{m}$). Figure 3b is a histological characterization showing the further development of the meristem toward the epidermis in St. John's wort hypocotyl cultured on TDZ-containing induction medium. (bar = $50 \mu\text{m}$). Figure 3c is a histological characterization showing the development of shoot primordia and the development of a

vascular connection (arrow) in St. John's wort hypocotyl cultured for 2 weeks on TDZ-containing induction medium. (bar = 166 μ m). Figure 3d is a histological characterization showing a well developed shoot (bar = 166 μ m) and vascular connections between the adventitious shoot bud and the parental tissue (arrow) in St. John's wort hypocotyl cultured for 18 days on TDZ-containing induction medium. (bar = 166 μ m)

Figure 4 shows several aspects of *Echinacea* as produced according to the method as described herein. Figure 4(a) illustrates callus formation and shoot regenerates appearing at the callus interface on petiole explants of *Echinacea purpurea* cultured on BAP-containing induction medium. Figure 4b shows individual shoot regenerants with well defined leaf initials on petiole explants of *Echinacea purpurea* cultured on BAP-containing induction medium after 33 days of culture. Figure 4c shows a late cotyledonary stage embryo on a BAP-induced petiole explant of *Echinacea purpurea*. Figure 4d shows complete plantlets grown from embryos regenerated on petioles of *Echinacea purpurea* cultured on BAP-containing induction medium and cultured on basal medium for 2 months.

Figure 5 shows several aspects of *Echinacea* as produced according to the method of the present invention. Figure 5(a) shows a histological characterization showing periclinal cell division in the subepidermal layers of the petiole explants of *Echinacea purpurea* at day 3 of culturing on induction medium containing BAP. (bar = 20 μ m). Figure 5(b) shows histological characterization showing formation of promeristomatic centres (arrows) in the callus tissue of petiole explants of *Echinacea purpurea* at day 14 of culturing on induction medium containing BAP. (bar = 680 μ m). Figure 5(c) shows histological characterization showing promeristomatic centres, having cells which were smaller in size and had a dense cytoplasm and prominent nuclei (arrows), observed on the callus tissue of the petiole explants of *Echinacea purpurea* by day 14 of culturing on induction medium containing BAP. (bar = 50 μ m). Figure 5(d) shows

histological characterization showing how the promeristomatic centres developed further to form dome shaped meristem zones which were well defined on the petiole explants of *Echinacea purpurea* cultured on induction medium containing BAP by day 21. (bar = 50 μ m). Figure 5(e) is a histological characterization showing development of a shoot meristem and leaf primordia on petiole explants of *Echinacea purpurea* after 21 days of culturing on induction medium containing BAP. (bar = 320 μ m). Figure 5(f) is a histological characterization showing a well developed shoot bud surrounded by leaf primordia formed on petiole explants of *Echinacea purpurea* after 28 days of culturing on induction medium containing BAP. Note that trichomes were observed to be associated with the leaf primordia and xylem elements were present at the base of the shoot bud. (bar = 166 μ m).

Figure 6 shows several aspects of *Echinacea* as produced according to the present invention. Figure 6 (a) shows a histological characterization showing a series of anticlinal and periclinal divisions leading to the formation of well defined protoderm regenerated from petiole explants of *Echinacea purpurea* at 14 days of culturing on induction medium containing BAP. (bar = 50 μ m). Figure 6(b) is a histological characterization showing a well developed heart-shaped somatic embryo with a fully formed protoderm regenerated from petiole explants of *Echinacea purpurea* at 21 days of culturing on induction medium containing BAP. (bar = 320 μ m).

Figure 7 shows a bar graph of the effect on zinc supplementation of the culture medium on accumulation of zinc in *Echinacea* plantlets after 30 days in culture.

Figure 8 is a bar graph showing accumulation of lithium in St. John's wort plantlets cultured on a medium supplemented with 0-200 mg/L lithium for 30 days.

Detailed Description of the Invention:

The present invention relates to methods for the micropropagation of phytopharmaceutical plants, and to plants obtained by using this method. Furthermore, the present invention relates to methods for the fortification of phytopharmaceutical plants with nutrients, minerals or other compounds and to plants obtained using these methods.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

As used herein, the term phytopharmaceutical plant means any plant that exhibits a beneficial or medicinal effect when administered via any means to a human or an animal. The beneficial or medicinal effect of the phytopharmaceutical plant may have been verified by laboratory or clinical study. A phytopharmaceutical plant may also comprises one or more active agents whose identity has been identified. Preferably the phytopharmaceutical plant is a medicinal plant, for example, but not limited to:

Achillea millefolium
Achyranthes bidentata
Aconitum napellus
Adonis aestivalis
Agastache mexicana
Agrimonia eupatoria
Agathosma betulina
Allium sp
Anchusa officinalis
Anemopsis californica

Angelica dahurica
Angelica polymorpha sinensis (A. sinensis)
Arnica Montana
Ammi visnaga
Arctostaphylos uva-ursi
Asclepias tuberosa
Astragalus membranaceus
Astragalus chinensis
Baphicacanthus cusia
Bixa orellana
Bupleurum falcatum
Brugmansia (Datura) spp.
Campanula rapunculus
Carum roxburgianum
Carum copticum
Cassia tora
Chamaelirium luteum
Chimaphila umbellata
Commiphora africana
Conium maculatum
Crithium maritimum
Datura metel (Datura alba)
Datura inoxia
Dracocephalum moldavica
Echinacea sp.
Eclipta alba (E. prostrata)
Ephedra nevadensis
Eriodictyon californicum
Eucommia ulmoides

Eupatorium perfoliatum
Filipendula vulgaris (F. hexapetala)
Gaultheria procumbens
Geum urbanum
Houttuynia cordata
Hydrocotyle asiatica (Centella asiatica)
Hypericum perforatum cv. Anthos
Inula helenium
Jatropha curcas
Leptospermum scoparium
Lespedeza capitata
Ligusticum porteri
Ligustrum lucidum
Lithospermum officinale
Lycium barbarum
Mucuna pruriens
Mandragora officinarum
Origanum dictamnus
Parietaria judaica (P. officinalis)
Phyllanthus emblica
Picrasma excelsa
Piniella ternate
Pogostemon patchouli
Polygonum multiflorum
Porophyllum ruderale ssp. macrocephalum
Prunella vulgaris
Pueraria lobata (P. thunbergiana)
Rauvolfia serpentina
Rivea corymbosa

Sanguinaria Canadensis
Satureja douglasii
Schizonepeta tenuifolia
Scutellaria baicalensis
Solanum xanthocarpum (S. surattense)
Sutherlandia frutescens
Tabebuia impetiginosa
Tanacetum parthenium
Tribulus terrestris
Trichosanthes kirilowii
Turnera diffusa
Voacanga africana
Withania somnifera

More preferably the plant is selected from the group consisting of St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea* sp., feverfew (*Tanacetum parthenium*), garlic (*Allium* sp.).

The present invention also pertains to the method of phytofortification. Phytofortification refers to the incorporation of one or more additives of interest within a plant, for example, one of the plants identified above, thereby rendering one or more additives in a bio-available form suitable for human or animal consumption. Preferably, the one or more additives of interest is selected from, but not limited to, an essential nutrient, vitamin, metabolite or combination thereof. Phytofortification utilizes the inherent ability of a plant or plantlet to uptake one or more additives from a growth medium. Plantlets are grown in a controlled environment and accumulate the nutrients in the consumable biomass. The process of phytofortification exploits the natural biological processes that convert inorganic nutrients and other additives into complex organic molecules within the plant tissues. This process renders nutrients and

additives amenable for efficient assimilation and increased the efficiency of adsorption by human or livestock digestive tracts through digestion of plant-based macromolecules. The bioavailability of the phytofortified additives and supplements in the plant products facilitates the utilization of the nutrients for nutritional and medicinal processes.

The present invention therefore provides a method for the phytofortification of *in vitro* or *in vivo* -grown phytopharmaceutical plants.

The term "additive" includes mineral elements, vitamins, metabolites and other nutrients as are known in the art. Examples of an additive, which are not to be considered limiting in any manner includes the mineral elements, boron, calcium, chloride, chromium, cobalt, copper, iron, lithium, iodine, magnesium, manganese, molybdenum, nickel, phosphorous, potassium, selenium, silicon, sodium, sulphur, tin, vanadium and zinc.

In vitro micropropagation

In general terms, one of the methods of this invention involves obtaining a sterile explant of a phytopharmaceutical plant and culturing it on an induction medium comprising a suitable concentration of at least one plant growth regulator that has cytokinin activity for a suitable period of time in order to produce regenerated tissue. The explant can be selected from any part of the plant including seeds, petiole, hypocotyl, cotyledon, stem and leaves. If the tissue is hypocotyl, it is preferably etiolated hypocotyl.

The plant growth regulator having cytokinin activity may be any such synthetic or natural plant growth regulator including for example, but not limited to, thidiazuron (TDZ; *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU

(N-(2-chloro-4pyridyl)-N(-phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6- γ,γ -dimethylallylamino purine). Preferably, the plant growth regulator having cytokinin activity is selected from thidiazuron (TDZ) and benzylaminopurine (BAP).

The concentration and duration of exposure of the sterile explant to the plant growth regulator(s) having cytokinin activity may vary depending on the species of the plant. A person skilled in the art can determine the concentration of growth regulator, and the duration of exposure by culturing an explant from the phytopharmaceutical plant at various concentrations of the plant growth regulator(s) and for various exposure times and determining the optimal conditions for the production of the desired amount of regenerated tissue. The regenerated tissue may be in the form of shoots, calli or somatic embryos. Most commonly the regenerated tissue is in the form of shoots. It is desired to use a concentration and duration of exposure of the plant growth regulator(s) having cytokinin activity which minimizes residual effects including a decrease in the number of regenerants, vitrification of the shoots and poor rooting of regenerants.

Following culture of the phytopharmaceutical plant explant on the medium comprising at least one plant growth regulator having cytokinin activity, the explant with regenerated tissue may be transferred to basal medium and cultured under standard conditions, as known to one of skill in the art, to form plantlets. Alternatively, the explant with regenerated tissues may be transferred to basal medium and subcultured for a further period of time to obtained the desired amount of regenerated tissue formed. Following this subculturing, the regenerated tissue may be transferred to either solid or liquid basal medium and cultured under standard conditions to form plantlets.

Examples of the media which may be used for this method, which are to be

considered non-limiting, are presented in Table 1:

Table 1

	Induction Medium	Solid Basal	Liquid Basal
pH	5.7	5.8	5.8
MS salts (Murashige & Skoog, 1962)	+	+	+
B5 Vitamins (Gamborg, 1968)	+	+	+
Sucrose	3%	3%	3%
Gellan Gum	0.3%	0.3%	---
Growth Regulator	Cytokinin activity	---	---

Sterile explants of phytopharmaceutical plants may be obtained using standard procedures, for example, but not limited to, seedlings grown from sterile seeds on water agar in darkness in a growth cabinet at a suitable temperature, for example in the range of about 22 to about 28 °C, preferably at about 24 °C, for a suitable period of time, for example about 10 to about 60 days. Seeds may be sterilized by immersion in alcohol, for example about 70 to about 95% ethanol, for a short period of time, for example 5 seconds to about 5 minutes, followed by soaking in a solution containing about 1 to about 3%, preferably 1.5%, (v/v), sodium hypochlorite (Javex), or other

suitable bleach, for a period of time, for example, but not limited to, about 15 to about 25 min, preferably about 17 to about 22 min. The bleach solution preferably also contains from about 1 to about 10 drops of Tween-20 per 100 mL of solution. Following this sterilization procedure the seeds are thoroughly rinsed with sterile deionized or distilled water.

Some seeds from phytopharmaceutical plants are particularly difficult to sterilize due to a high amount of fungal contamination. In these instances a biostatic agent such as plant preservative mixture (PPM) may be added to the water agar during culture. The amount of the biostatic agent to be used can be determined by culturing the seed or explant on water agar with varying amounts of the biostatic agent and determining the lowest concentration that would be biostatic to fungal growth and still allow seed germination or explant regeneration.

An alternate method for the sterilization of explants may involve surface sterilization of field or greenhouse-grown plant material. With this method, tissue is excised from the intact plant and immediately immersed in sterile water. A similar sterilization protocol would then be followed in which the tissue is rinsed with an alcohol, from about 70 to about 90%, for 30 seconds to 5 minutes, followed by immersion in a bleach solution containing from about 1 to about 10 drops of Tween-20 per 100 mL for 15-40 minutes. Preferably the alcohol is ethanol. Following this sterilization protocol, the tissue should be thoroughly rinsed in sterile deionized or distilled water and cultured on induction medium.

In one embodiment of the present invention, there is provided a method for the *in vitro* micropropagation of St. John's wort. *De novo* shoot regeneration can be effectively induced by culturing sterile explants of St. John's wort on a medium containing at least one plant growth regulator having cytokinin activity as defined above. Preferably, the plant growth regulator having cytokinin activity is TDZ. The

tissue source for this method can be any suitable sterile tissue from St. John's wort, for example, but not limited to, seeds, stems, petioles, hypocotyl, cotyledon and leaves. Preferably the tissue is etiolated hypocotyl. The concentration of plant growth regulator having cytokinin activity may be in the range of from about 0.001-25 $\mu\text{mol} \cdot \text{L}^{-1}$, preferably from about 1.5 to about 20 $\mu\text{mol} \cdot \text{L}^{-1}$, more preferably about 3 to about 15 $\mu\text{mol} \cdot \text{L}^{-1}$, even more preferably about 4 to about 10 $\mu\text{mol} \cdot \text{L}^{-1}$. The explant is exposed to the inducing media comprising the plant growth regulator having cytokinin activity from about 1 to about 15 days, preferably from about 6 to about 12 days, more preferably from about 8 to about 10 days.

Following initial culture on the induction medium, the explants of St. John's wort may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum amount of regenerated shoots/explant. The period of time for subculture will depend on the number of shoots/explant desired and the size restrictions of the culture flask. Optimally, the explants of St. John's wort may be subcultured for about 1 to about 15 days, preferably from about 5 to about 12 days, more suitably from about 8 to about 10 days. The regenerated shoots may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots may be transferred to culture boxes containing solid basal medium or to flasks containing liquid basal medium. At least 90% of the regenerants develop into mature plantlets which are morphologically similar to seed grown plants raised in the greenhouse. Alternatively, the explants and/or regenerated shoots may be transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

The culturing of St. John's wort hypocotyl segments on medium in the absence of exogenous growth regulators, produced a large number of explants that formed 1 or 2 adventitious roots. This spontaneous root formation is likely indicative of a high level of endogenous auxin in the etiolated hypocotyls. Supplementation of the culture

medium optimized with a plant growth regulator having cytokinin activity suppressed this response. Without wishing to be bound by theory, this effect may be a result of an alteration in the auxin-to-cytokinin ratio within the tissues.

Also described herein is a method for the *in vitro* micropropagation of *Echinacea* sp., in particular, *Echinacea purpurea*. Sterile explants of *Echinacea* can be cultured on induction medium in the presence of at least one plant growth regulator having cytokinin activity as defined above. Preferably, the plant growth regulator having cytokinin activity is selected from TDZ and BAP. The concentration of plant growth regulator having cytokinin activity may be in the range of from about 0.001 to about 25 $\mu\text{mol} \cdot \text{L}^{-1}$, preferably from about 0.5 to about 20 $\mu\text{mol} \cdot \text{L}^{-1}$, more preferably about 1 to about 15 $\mu\text{mol} \cdot \text{L}^{-1}$.

The tissue source for this method can be any suitable sterile tissue from *Echinacea*, for example but not limited to seeds, stems, petioles, hypocotyl, cotyledon and leaves. Most preferable the tissue is petiole.

The explant is exposed to the inducing media comprising the plant growth regulator having cytokinin activity from about 1 to about 50 days, preferably from about 6 to about 40 days, more preferably from about 10 to about 35 days. If the induction medium also contains an auxin, the time for exposure to the growth regulator may need to be on the higher side of this range, for example up to about 25 to about 40 days.

Histological observations revealed that regeneration in petiole cultures of *Echinacea* under the conditions used herein occurred primarily as a result of *de novo* shoot formation from callusing tissue. Other culture conditions may provide regenerated shoots directly. Histological evidence indicates that some somatic embryos were also formed during the culture of *Echinacea* petiole explants on induction

medium containing BAP. These somatic embryos may be transferred to basal medium and cultured under suitable conditions to form plantlets.

Following initial culture on the induction medium, the explants of *Echinacea* may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum amount of regenerated tissue/explant. The period of time for subculture will depend on the amount of regenerated tissue/explant desired and the size restrictions of the culture flask. Optimally, the explants of *Echinacea* may be subcultured for about 1 to about 15 days, preferably from about 5 to about 12 days, more preferably from about 8 to about 10 days. The regenerated tissue may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots or somatic embryos may be transferred to culture boxes containing solid basal medium or to flasks containing liquid basal medium. Under the conditions defined above, about 70% of the regenerants develop into mature plantlets. These plantlets are morphologically similar to seed grown plants raised in the greenhouse. Alternatively, the explants or regenerated tissue may be transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

Indirect morphogenesis is the formation of callus from explants which subsequently results in shoots or somatic embryogenesis (Sharp 1980). Callus formation and cell enlargement in tissues may be indicative of high endogenous auxins (Skoog & Miller, 1957). While not wishing to be limited by theory, the formation of callus at the cut ends of the petioles on the control plates as well as elongation of the explants appears to indicate high levels of endogenous auxins present in petioles of *Echinacea purpurea*. In *Echinacea* petiole cultures, adjacent cells were stimulated to follow different regenerative routes in the presence of the same cytokinin. While not wishing to be bound by theory, these data may indicate that the endogenous metabolites and growth regulators in individual cells may be different, thereby either

predetermining or effecting the fate of the cells during regeneration.

The present invention also provides a method for the *in vitro* micropropagation of Huang-qin (*Scutellaria baicalensis*). Sterile explants of Huang qin can be cultured on induction medium in the presence of at least one plant growth regulator having cytokinin activity. Preferably, the plant growth regulator having cytokinin activity is selected from TDZ and BAP. Most preferably, the plant growth regulator having cytokinin activity is TDZ. The concentration of plant growth regulator having cytokinin activity may be in the range of from about 0.001 to about 25 $\mu\text{mol} \cdot \text{L}^{-1}$, preferably from about 0.05 to about 20 $\mu\text{mol} \cdot \text{L}^{-1}$, more preferably from about 1.5 to about 20 $\mu\text{mol} \cdot \text{L}^{-1}$, and most preferably about 2.0 to about 20 $\mu\text{mol} \cdot \text{L}^{-1}$.

The tissue source for this method can be any suitable sterile tissue from Huang-qin, for example seeds, stems, petioles, hypocotyl, cotyledon and leaves, preferably the tissue is seed, hypocotyl or stem. Most preferably, the tissue is seed.

The explant is exposed to the inducing media comprising the plant growth regulator having cytokinin activity from about 1 to about 30 days, preferably from about 10 to about 25 days, more preferably from about 14 to about 20 days.

Following initial culture on induction medium, the explants of Huang-qin may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum number of shoots/explant. The period of time for subculture will depend on the number of shoots/explant desired and the size restrictions of the culture flask. Optimally, the explants of Huang-qin may be subcultured for about 1 to about 15 days, preferably from about 5 to about 12 days, more preferably about 8 to about 10 days. The regenerated shoots may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots may be transferred to culture boxes containing solid basal medium

or to flasks containing liquid basal medium. Under the conditions described herein, about 90% of the regenerants develop into mature plantlets which are morphologically similar to seed grown plants raised in the greenhouse. Alternatively, the explants and/or regenerated shoots may be transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

It is another embodiment of the present invention to provide a method for the *in vitro* micropropagation of feverfew (*Tanacetum parthenium*). Sterile explants of feverfew can be cultured on induction medium in the presence of at least one plant growth regulator having cytokinin activity. Preferably, the plant growth regulator having cytokinin activity is TDZ. The concentration of plant growth regulator having cytokinin activity may be in the range of from about 0.001 to about 25 $\mu\text{mol} \cdot \text{L}^{-1}$, preferably 0.5 to about 20 $\mu\text{mol} \cdot \text{L}^{-1}$, more preferably from about 1.5 to about 15 $\mu\text{mol} \cdot \text{L}^{-1}$, and most more preferably about 2.0 to about 8 $\mu\text{mol} \cdot \text{L}^{-1}$.

The tissue source for this method can be any suitable sterile tissue from feverfew, for example seeds, stems, petioles, hypocotyl, cotyledon and leaves. The use of leaf, stem, petiole and hypocotyl is preferred.

The explant is exposed to the inducing media comprising the plant growth regulator having cytokinin activity from about 1 to about 50 days, preferably from about 10 to about 40 days, more preferably from about 20 to about 35 days.

Following initial culture on the induction medium, the explants of feverfew may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum number of shoots/explant. The period of time for subculture will depend on the number of shoots/explant desired and the size restrictions of the culture flask. Optimally, the explants of feverfew may be subcultured for about 1 to about 15 days, preferably about 5 to about 12 days, more

preferably about 8 to about 10 days. The regenerated shoots may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots may be transferred to culture boxes containing solid basal medium or to flasks containing liquid basal medium. Under these conditions, about 90% of the regenerants develop into mature plantlets which are morphologically similar to seed grown plants raised in the greenhouse. Alternatively, the explants and/or regenerated shoots may be transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

The methods developed herein for the *in vitro* micropropagation of phytopharmaceutical plants are ideal for adaptation for use in a bioreactor-type system such as that shown in Figure 1. The bioreactor is a large scale, sterile vessel for growth of plant cells and intact plants in culture media and in a controlled environment. Usually the growth media is re-circulated through the culture vessel by a series of peristaltic pumps and media is aerated to ensure rapid and prolific growth of the cultured tissue. In this way, the growth of the plants or cell cultures can be maintained within the closed system in perpetuity. The bioreactor system can be optimized to produce intact plantlets for whole plant preparations or the media components can be altered for the efficient production of various secondary metabolites that serve as the active ingredients in the phytopharmaceutical plant. The use of the bioreactor system will provide phytopharmaceutical manufacturers with a year-round supply of high quality, consistent plant material.

Production of fortified phytopharmaceutical plants

In general terms, the method for the production of fortified phytopharmaceutical plants involves obtaining a sterile, rapidly growing plantlet, seedling or explant of a phytopharmaceutical plant and subculturing it on a medium supplemented with a nutrient mineral element. If an explant is used, it may be selected

from any regenerating explant or part of the plant including seeds, petiole, hypocotyl, cotyledon, stem, root and leaves. Preferably, the sterile explant is cultured on a medium containing at least one growth regulator having cytokinin activity under conditions suitable to form regenerated tissue as defined above.

The concentration and duration of exposure of the sterile plantlet or culture to the mineral or nutrient element(s) will vary depending on the species of the plant. A person skilled in the art can determine these values by exposing a rapidly growing culture of the phytopharmaceutical plant at various concentrations of mineral or nutrient element(s) and for various exposure times and determining the optimal conditions for the production of the maximum amount of fortified tissue.

Examples of the media which may be used for the method, which are to be considered non-limiting, are presented in Table 1 (above)

Sterile plantlets of phytopharmaceutical plants may be obtained as described above. However, the present invention also includes an alternate method for the fortification of phytopharmaceutical plants involving the subculture of plantlets or greenhouse-grown plant material in a controlled environment nutrient recycling system. With this method, the plantlets would be grown in on a standardized solution supplemented with the nutrient or mineral element.

The methods as described herein can be used for the phytofortification of plant species with any additive of interest, for example, but not limited to zinc. Zinc deficiency is one of the leading causes of limited growth rate, loss of appetite, skin lesions, delayed wound healing, hypogonadism, delayed sexual maturation and impaired immune responses in mammals (Whittaker, 1998). The preparation of a phytofortified product containing bioavailable zinc provides a new class of nutrient supplement for balancing and maintaining optimal nutritional status for both human

and livestock applications. It is to be understood that any phytopharmaceutical plant may be phytofortified using the method of the present invention.

In a preferred embodiment, the phytofortification method described herein can be used to supplement zinc within a desired phytopharmaceutical plant, for example, but not limited to *Echinacea*, during the growth process. The zinc can be supplied either in the growth medium of *in vitro* grown *Echinacea* seedlings or *Echinacea* plants grown in greenhouse hydroponic systems. The maintenance of an optimized level of zinc and minimization of the phytic acid of the plants ensures the high bioavailability of the zinc supplemented *Echinacea* product.

Example 5 described below outlines the phytofortification of *Echinacea* with zinc. The method involves preparing *Echinacea* petiole explants harvested from 1 month-old sterile seedlings on a media supplemented with a cytokinin as described above. Using liquid cultures, *Echinacea* roots, of a size suitable for commercial use were obtained in about 4 months, as compared to roughly 3 years of growth under field conditions in Canada. Supplementation of the liquid culture medium with zinc resulted in the accumulation of the nutrient in *Echinacea* tissues over a period of 3 to 4 weeks.

Phytofortification of plant species with lithium is also described herein. In a preferred embodiment, the method can be used to fortify St. John's wort with lithium, however, other phytopharmaceutical plants may also be fortified with lithium as described herein.

The combination of lithium with St. John's wort, a mediator of mammalian serotonin metabolism, offers a new pharmaceutical approach to the treatment of depression. The concentration of lithium in the product can be tightly controlled at the plant level. By providing lithium in a bio-available form, the toxicity that can result

from the accumulation and impaired excretion of lithium salts when administered on their own may be reduced or eliminated.

A protocol for the induction of shoot organogenesis on St. John's wort explants results in the clonal production of more than 40 *de novo* shoots on a 1 cm tissue segment. Masses of genetically consistent, sterile plant material were grown in a controlled environment for 2 months. Supplementation of the culture medium with lithium resulted in the significant accumulation of the mineral within 3-4 weeks as described in Example 6.

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner

Examples:

General Procedures:

Statistical analyses:

The design of all experiments was a complete randomized block and treatments consisted of five replications. All the experiments were repeated at least twice and the data were analyzed using SAS Version 6.12 (SAS Inc., 1995). Significant differences between means were assessed by a Student-Neuman-Keulls means separation test at $P \leq 0.05$.

Light microscopic studies:

The explants were harvested at weekly intervals over the 4 week incubation period and fixed immediately in formalin:acetic acid:alcohol (FAA: 5:5:90; V/V) and stored at 4°C. The tissues were dehydrated with a graded ethanol series followed by paraffin embedding. Eight μm thin sections were cut using an ultra microtome (Porter-Blum Ultra microtome MT-1, Ivan Sorvall Inc., Connecticut, USA). The sections were stained (Johnson, 1942) with alcian green and counter stained with safranine and observed under a light microscope (Zeiss, Germany).

Example 1: Micropropagation of St. John's wort

St. John's wort seeds were sterilized by immersing in a 70% ethanol solution for 5 s, followed by an immersion in a 30% solution of 5.4% sodium hypochlorite (Lilo Products, Hamilton, Ontario) in water with one drop of Tween 20 per 500 mL for 20 min, and a three times rinse in sterile distilled water. Sterile seeds were germinated and maintained on water agar ($8 \text{ g} \cdot \text{L}^{-1}$) for 16 days in darkness in a growth cabinet at 24 °C. Hypocotyl sections were excised from sterile etiolated seedlings and cultured on a medium containing MS medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al 1968), $30 \text{ g} \cdot \text{L}^{-1}$ sucrose and $3 \text{ g} \cdot \text{L}^{-1}$ gellan gum (Gelrite, Schweitzerhall Inc., South Plainfield, NJ, USA). Varying levels (0, 5, 10, 15 and 20 $\mu\text{mol} \cdot \text{L}^{-1}$) of TDZ and BAP (Sigma Chemical Co., St. Louis, USA) were incorporated into the basal culture medium in a series of experiments. Each experiment consisted of six explants per plate and 20 plates per plant growth regulator treatment. For determination of the optimal duration of exposure of the explants to the medium containing TDZ, five plates of each treatment were subcultured onto MS basal medium at days 3, 6, 9 and 12. All cultures were incubated in a growth cabinet with a 16 hour photoperiod under cool white light at $40\text{-}60 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Regeneration was quantified after 18 and 23 days of culture.

Hypocotyls cultured on a medium without exogenous growth regulators produced an average of 0.8 roots/explant while rooting was not observed on explants cultured on medium containing TDZ (Table 2). *De novo* shoot organogenesis was induced on etiolated hypocotyl segments cultured on media supplemented with TDZ after 18-21 days (Figure 1(a)). Explants which remained on the TDZ medium throughout the treatment period exhibited symptoms of browning and the regenerated shoots appeared stunted and malformed as compared to those which developed on explants transferred to basal medium following a brief exposure to TDZ. Supplementation of the induction medium with TDZ alone resulted in the induction of significantly more regeneration than any other combination of growth in these experiments. Under the conditions described herein, the optimal concentration of TDZ for induction of shoot organogenesis on etiolated St. John's wort hypocotyls was $5 \mu\text{mol} \cdot \text{L}^{-1}$. The induction of regeneration was effected by the duration of exposure to TDZ. Under the conditions defined above, the optimal culture period on TDZ-supplemented medium was 9 days with subculture onto basal media for a further 9 day period. Varying the culture conditions may change these optimal values. The protocol described herein produced a mean of 54.0 shoots/explant in 18 days (Table 2). Regenerated shoots, transferred into culture boxes containing the basal medium, formed roots and whole plantlets within 2 months (Fig. 2(b) and (c)). Similarly, the regenerated shoots formed vigorously growing plantlets in flasks containing liquid basal medium. More than 95% of the regenerants developed into mature plantlets which were morphologically similar to seed grown plants raised in the greenhouse.

The sections were observed under a light microscope to investigate the histological process of adventitious shoot development in St. John's wort hypocotyl cultures grown on induction medium ($5 \mu\text{mol} \cdot \text{L}^{-1}$ TDZ). Abundant meristematic zones developed at the hypodermal layers of the hypocotyl as early as 7 days after culture initiation (Fig. 3(a), arrows). The meristematic zones consisted of cells which were small in size with dense cytoplasm and a prominent nuclei (Fig. 3(a)). After 9

days of culture, these meristematic zones further differentiated and the development progressed toward the epidermis (Fig. 3(b)). In observations made after two weeks of culture, the development of shoot primordia was clearly visible (Fig. 3(c), arrows). The shoot primordia developed into fully developed shoots with vascular connections between the vascular bundle of the explant and the developing regenerant by day 18 (Fig. 3(d)).

FIG. 3(a) through 3(d) are micrographs showing the development of shoot primordia from explants. FIG. 3(a) shows an explant with a vascular bundle. FIG. 3(b) shows the explant after 7 days of culture, with shoot primordia developing. FIG. 3(c) shows the explant after 14 days of culture, with shoot primordia clearly visible (arrows). FIG. 3(d) shows the explant after 18 days of culture, with fully developed shoots and vascular connections.

Table 2: Effects of different concentrations of TDZ and duration of culture on TDZ supplemented medium on regeneration of St. John's wort hypocotyl explants. Statistical differences assessed by the Student Newman-Kuells mean separation test.

[TDZ] $\mu\text{mol} \cdot \text{L}^{-1}$)	Day transferred onto basal medium	Number of Shoots/hypocotyl	Number of Roots/hypocotyl
0	0	4.6 ^c	0.8 ^a
5	6	27.9 ^c	0.3 ^{bc}
5	9	54.0 ^a	0.0 ^c
5	12	42.8 ^{ab}	0.0 ^c
10	3	11.5 ^{dc}	0.2 ^{bc}
10	6	13.5 ^{dc}	0.0 ^c
10	9	47.6 ^{ab}	0.0 ^c
10	12	46.3 ^{ab}	0.0 ^c
15	3	12.5 ^{de}	0.1 ^{bc}
15	6	22.6 ^{cd}	0.0 ^c
15	9	47.0 ^{ab}	0.0 ^c
15	12	30.2 ^c	0.0 ^c
20	3	20.7 ^{cd}	0.3 ^b
20	9	45.2 ^{ab}	0.0 ^c
20	12	39.0 ^b	0.0 ^c

abcde Values within a column with different superscripts are significantly different ($P < 0.05$).

Example 2: Micropropagation of *Echinacea purpurea*

Echinacea purpurea achenes were sterilized by immersing in 70% ethanol for 30 sec, soaking in 5.4% sodium hypochloride (Javex) in water with one drop of Tween 20 per 100 ml for 18 min and rinsing three times in sterile deionized water. Due to a

high amount of fungal contamination present in the seed coat of *Echinacea purpurea* achenes, PPM was included in the water agar to obtain sterile seedling germination for culture. Sterile seeds were germinated on water agar ($8 \text{ g} \cdot \text{L}^{-1}$) with $3 \text{ ml} \cdot \text{L}^{-1}$ plant preservation mixture (PPM) in a growth cabinet in 24 hour darkness at 24°C for 14 days. Different concentrations of PPM were included in with the water agar (1, 2, 3, 4 and $5 \text{ ml} \cdot \text{L}^{-1}$) to determine the lowest amount which would be biostatic to fungal growth. A concentration of $3 \text{ ml} \cdot \text{L}^{-1}$ PPM was found to be suitable for germination of *Echinacea* seeds under the present conditions as defined herein. This concentration may vary depending on the culture conditions.

Germinating seedlings were cultured onto MS medium (Murashige & Skoog, 1962) with B5 vitamins (Gamborg et al 1968), $30 \text{ g} \cdot \text{L}^{-1}$ sucrose and $3 \text{ g} \cdot \text{L}^{-1}$ gelrite in Magenta boxes. Petiole explants, 2 cm in length were excised from 4 week old sterile *Echinacea purpurea* plants and cultured onto induction medium comprising MS media supplemented with thidiazuron (TDZ) (0.5, 1, 5 and $10 \mu\text{mol} \cdot \text{L}^{-1}$) or BAP (1, 2.5, 5, 7.5, 10, 12.5 and $15 \mu\text{mol} \cdot \text{L}^{-1}$). TDZ was added to the media at concentrations of 0.5 and $1.0 \mu\text{mol} \cdot \text{L}^{-1}$ in combination with indole acetic acid (IAA, an auxin) at concentrations of 5 and $10 \mu\text{mol} \cdot \text{L}^{-1}$. Explants cultured on media containing TDZ and IAA were subcultured onto MS basal media after 23 days while those cultured on media containing higher concentrations of TDZ were subcultured onto MS basal media after 4 and 8 days. All treatments consisted of 10 plates per treatment and 6 explants per plate. Treatments were incubated in a growth cabinet with a 16 hour photoperiod under cool white light at $40\text{-}60 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Regeneration was quantified after 25 and 33 days for all petiole cultures and roots after 33 and 42 days of culture. The resulting regenerants were excised from petioles and subcultured onto MS basal media in test tubes for germination after 32 days of culture.

Supplementation of the culture medium with BAP as the sole growth regulating compound induced *de novo* shoot formation at all concentrations (Fig. 4, Table 3). In addition to regeneration, supplementation of the culture medium with BAP was also observed to induce callus formation and elongation of the petioles. When IAA was combined with TDZ or the medium was supplemented with TDZ alone, the formation of *de novo* regenerants was observed (Table 4).

Table 3: Effects of the cytokinin BAP on regeneration of *Echinacea purpurea* petiole explants. Statistical differences assessed by the Student Newman-Kuells mean separation test after 33 days of culture.

BAP Concentration ($\mu\text{mol}\cdot\text{L}^{-1}$)	Number of regenerants/petiole
0	0.0 ^c
1	5.4 ^{ab}
2.5	8.1 ^a
5	6.6 ^{ab}
7.5	5.2 ^{ab}
10	3.9 ^b
12.5	5.2 ^{ab}
15	5.5 ^{ab}

^{abc} Values within a column with different superscripts are significantly different ($P < 0.05$).

Table 4: Effects of the auxin IAA and TDZ on production of somatic embryos and roots of *Echinacea purpurea* petiole explants. Statistical differences assessed by the Student Newman-Kuells mean separation test after 33 days of culture.

IAA Concentration ($\mu\text{mol} \cdot \text{L}^{-1}$)	TDZ Concentration ($\mu\text{mol} \cdot \text{L}^{-1}$)	Number of regenerants/petiole	Number of roots/petiole
0	0	0.0 ^b	0.0 ^b
0	0.5	3.5 ^a	0.0 ^b
0	1.0	3.2 ^a	0.0 ^b
5	0	0.1 ^b	4.4 ^a
5	0.5	3.3 ^a	0.0 ^b
5	1.0	2.4 ^a	0.0 ^b
10	0	0.6 ^b	4.5 ^a
10	0.5	4.9 ^a	0.1 ^b
10	1.0	3.3 ^a	0.0 ^b

^{ab} Values within a column with different superscripts are significantly different ($P < 0.05$).

Petiole explants cultured on MS media + 5 $\mu\text{mol} \cdot \text{L}^{-1}$ BAP were harvested at 0, 3, 5, 7, 14, 21, 28 and 35 days after culture initiation. Samples were immediately fixed in formalin/glacial acetic acid and 50% ethanol (FAA) mixture (5:5:90 v/v/v). Proper and rapid fixation of the sample was ensured by vacuuming the samples at -20 kPa for 10 min. The samples were then dehydrated through a graded tertiary butanol series and embedded in paraffin wax. Transverse 8 μm thin sections were cut using an ultra

microtome (Porter-Blum ultra microtome MT-1, Ivan Sorvall Inc., Connecticut, USA) and stained with alcian green and safranin (Jensen, 1962). The sections were observed under a compound light microscope (Zeiss, Germany).

Histological observations revealed that regeneration in petiole cultures of *Echinacea* occurred primarily as a result of *de novo* shoot formation from callusing tissue. After 3 to 7 days in culture, the epidermal and subepidermal layers of the petiole explant started to divide (Fig. 5(a)) and formed a compact mass of callus tissue (Fig. 5(b)). This callus tissue consisted of numerous meristomatic zones (Fig. 5(a) - arrows, Fig. 5(c)). The cells of these meristematic zones were small in size with dense cytoplasm and a prominent nuclei. These meristematic zones further underwent differentiation and formed a dome shaped shoot meristem by day 21 (Fig. 5(d) - arrows). The shoot meristem developed leaf primordia (Fig. 5 (e),(f) - arrows) and eventually formed shoot buds after 21 days. A well developed shoot bud consisted of a dome shaped shoot meristem surrounded by a few leaf primordia (Fig. 5(f)). The leaf primordia had well developed trichomes. Vascular elements were observed sporadically dispersed within the callus, mainly near the base of the shoot buds (Fig. 5(f) - arrows).

In addition to the shoot organogenesis, there was evidence of somatic embryogenesis in the histological examination of the petiole cultures. Proembryo like structures were observed in the subepidermal layers of the petiole as early as 14 days of culture (Fig. 6(a) - arrows). These proembryos appeared globular in shape, multicellular and were surrounded by a single outer wall. The proembryos later differentiated into heart shaped somatic embryos (Fig. 6(b)). These somatic embryos may be transferred to basal medium and cultured under conditions to form plantlets.

Example 3: Micropropagation of Huang-qin

Seed culture:

Huangqin (*Scutellaria biacalensis*) seeds were surface sterilized by dipping in 95% ethanol for 30s, then immersing in 1.5% sodium hypochlorite containing Tween-20 (2 drops per 100 ml solution) for 18 min and then rinsed 3 times in sterile distilled water. Five seeds were cultured aseptically in each Petri dish containing 25 ml of induction medium, which consisted of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al, 1968), $30\text{g}\cdot\text{L}^{-1}$ sucrose, $3\text{g}\cdot\text{L}^{-1}$ gelrite and amended with various concentration of TDZ and 4ml/l PPM (for killing fungus in seeds). The medium was adjusted to pH 5.75 before autoclaving at $121\text{ }^{\circ}\text{C}$, $1.4\text{ kg}\cdot\text{cm}^{-2}$, for 20 min. The Petri dishes were sealed with Parafilm and incubated in a growth chamber at $24 \pm 2^{\circ}\text{C}$ with a 16 h photoperiod provided by fluorescent tubes at $30\text{-}35\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Huang-qin seeds began to germinate after 7 days of culture. A few seedlings regenerated shoots from crown after 10 days of culture. On day 14, all germinating seedlings on the medium with TDZ began to develop *de novo* shoots. The seedlings cultured on the medium with TDZ at $2.5\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ had an average of 19 shoots per seedling, while the seeds germinated on the medium without TDZ had an average of 2 shoots per seedling. The medium containing 5.0, 7.5 and $10\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ also showed shoot formation (Table 5).

Table 5: Effect of TDZ on Induction of Shoot Formation in Huang-qin Seedlings After 14 Days of Culture

TDZ Concentration ($\mu\text{mol} \cdot \text{L}^{-1}$)	Number of Shoots/Seedling
0.0	2.32 ^c
2.5	19.85 ^a
5.0	12.05 ^b
7.5	17.40 ^{ab}
10.0	17.07 ^{ab}
20.0	17.25 ^{ab}

^{abc} Values within a column with different superscripts are significantly different ($P < 0.05$).

Hypocotyl culture:

Huang-qin seeds were sterilized as described above and aseptically cultured in Petri dishes containing 25 mL of 0.8% water agar with 4 mL/L PPM. Cultures were incubated in the dark at 24 °C for 14 days for germination. Six etiolated hypocotyl segments (about 0.5 cm) were excised from the seedlings and cultured on an induction medium comprising MS salts, B5 vitamins, 3% sucrose, 0.3% gelrite and various concentrations of TDZ (0, 2.5, 5, 7.5 and 10.0 $\mu\text{mol} \cdot \text{L}^{-1}$). Cultures were incubated in a growth chamber at 24 ± 2 °C with a 16 h photoperiod provided by fluorescent tubes at 30-35 $\mu\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 14 days.

Huang-qin hypocotyls on the MS basal medium in the absence of TDZ changed color to light purple after 7 days culture. After 10 days, they began to regenerate one or two adventitious shoots. After 14 and 18 days, shoot length increased quickly but no significant increase in shoot number per explant. Hypocotyls cultured on induction medium with TDZ at 2.5, 5.0, 7.5, and 10 $\mu\text{mol} \cdot \text{L}^{-1}$ appeared swollen and developed a green color after 7 days of culture. After 10 days, hypocotyls in these treatments began to form shoots on the swollen tissue. The treatment with TDZ at 7.5 $\mu\text{mol} \cdot \text{L}^{-1}$ induced significantly more shoots as compared with the control (Table 6). On day 14 and day 18, the number of shoots per explant on all the TDZ treatments rapidly increased. All explants on the medium containing TDZ formed shoots with an average of 8-11 shoots per explant while the explants cultured on medium containing no plant growth regulator had an average of 1 shoot per explant (Table 6).

Table 6: Effect of TDZ on Induction of Shoot Regeneration on Huang-qin Etiolated Hypocotyls

TDZ Concentration ($\mu\text{mol} \cdot \text{L}^{-1}$)	Number of Shoots on Day 10	Number of Shoots on Day 14	Number of Shoots on Day 18
0.0	1.03 ^b	1.08 ^b	1.50 ^b
2.5	1.96 ^b	9.67 ^a	11.06 ^a
5.0	1.53 ^b	9.39 ^a	9.44 ^a
7.5	2.40 ^a	8.00 ^a	8.42 ^a
10	2.00 ^b	8.17 ^a	9.33 ^a

^{ab} Values within a column with different superscripts are significantly different ($P < 0.05$).

Epicotyl culture:

For determination of the effect of TDZ on Huang-qin epicotyl tissues, stem segments (about 1.0 cm long) from sterile seedlings grown as described above on MS basal medium for 20 days were excised and culture on induction medium. The concentrations of TDZ tested were 0, 2.5, 5.0, 7.5 10 and 20 $\mu\text{mol} \cdot \text{L}^{-1}$. Induction cultures were incubated in a growth chamber at 24 ± 2 °C with a 16 h photoperiod provided by fluorescent tubes at 30-35 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ from 14 days. In all experiments, each treatment had 5 replicates and each experiment was repeated at least twice. Regeneration of shoots was observed after 10 days, 14 days and 18 days of culture and

quantified at 14 days of culture. The results for the quantification at 14 days are summarized in Table 7.

Huang-qin stems cultured on the medium containing TDZ began to swell at the edges of cuts and formed callus that appeared green in color by day 7. Regeneration of shoots was observed after 10 days of culture. On day 14, shoots had formed on all explants exposed to TDZ. The explants on the medium with TDZ had an average of 12-14 shoots per stem segment while explants cultured in the absence of growth regulators had an average of 3 shoots per explant.

Table 7: Effect of TDZ on Induction of Shoot Formation in Huang-qin Stem Explants Quantified on Day 14

TDZ Concentration ($\mu\text{mol} \cdot \text{L}^{-1}$)	Number of Shoots/Explant
0.0	3.54 ^c
2.5	13.61 ^a
5.0	14.19 ^b
7.5	14.58 ^{ab}
10.0	13.78 ^{ab}
20.0	12.61 ^{ab}

^{abc} Values within a column with different superscripts are significantly different ($P < 0.05$).

Example 4: Micropropagation of Feverfew

Preparation of the explants:

Mature feverfew (*Tanacetum parthenium*) were used for all of the experiments. Seeds were carefully selected for uniformity and surface sterilized by immersion in 70% (v/v) ethanol for 3 min., followed by a 20 min. soak in 1.5% (v/v) sodium hypochlorite in water containing 2 drops of Tween-20 per 100 mL and 5 rinses with sterile deionized water. Sterilized seeds were individually cultured in 50 mL glass tubes containing 10 mL of water agar with 3 mL/L of PPM. Seeds were germinated in the dark at 24 °C for the first 7 days and then moved to the light (30-35 (E m⁻²s⁻¹, 16 h photoperiod) were harvested after 2 months and cultured on a regeneration induction medium.

Regeneration induction medium:

Feverfew explants were cultured on an induction medium containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1965) and 30 g/L sucrose with thidiazuron (TDZ) or benzylaminopurine (BAP) at 0, 5, 10, 15, 20, 25 or 50 (mol/L. The pH of the medium was adjusted to 5.75 and 0.3% Gelrite (Scott Laboratories, Carson, USA) was included as the gelling agent prior to autoclaving at 1.4 Kg/cm² for 20 min. Regeneration was induced on stem, leaf and shoot explants cultured on an induction medium containing TDZ. The optimal level of TDZ supplementation for induction of regeneration of feverfew under the present conditions was 5 (mol/L. This value may vary depending on the conditions used.

Incubation of the cultures:

After 1 month, regenerated shoot cultures were transferred to a basal medium containing MS salts, B5 vitamins and 3% sucrose for further development. The

regenerated shoots formed roots and complete plantlets within 2 months of culture on solid basal medium. As well, prolific regeneration of plantlets was observed in cultures transferred to liquid basal medium. The optimal duration of exposure of feverfew explants to liquid basal medium was assessed in a temporary immersion bioreactor system. In this system, 30 day-old explants with prolific shoot regeneration were transferred to a sterile bioreactor flask. Cultures were incubated in the growth room at $35 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and liquid basal medium was pumped into the flask and drained from the flask at six hour intervals. The flask environment was aerated with a constant flow of sterile air throughout the incubation period. With this protocol, massive proliferation of feverfew plantlets was achieved in a 30 day period.

Example 5: Phytofortification of *Echinacea* with zinc

Echinacea achenes were sterilized by immersing in 70% ethanol for 30 sec, soaking in 5.4% sodium hypochloride (Javex) in water with one drop of Tween 20 per 100 ml for 18 min and rinsing three times in sterile deionized water. Due to a high amount of fungal contamination present in the seed coat of *Echinacea purpurea* achenes, PPM was included in the water agar to obtain sterile seedling germination for culture. Sterile seeds were germinated on water agar ($8 \text{ g} \cdot \text{L}^{-1}$) with $3 \text{ ml} \cdot \text{L}^{-1}$ plant preservation mixture (PPM) in a growth cabinet in 24 hour darkness at 24°C for 14 days. Different concentrations of PPM were included in with the water agar (1, 2, 3, 4 and $5 \text{ ml} \cdot \text{L}^{-1}$) to determine the lowest amount which would be biostatic to fungal growth. A concentration of $3 \text{ ml} \cdot \text{L}^{-1}$ PPM was found to be the optimal concentration for germination of *Echinacea* seeds under the present conditions. This concentration may vary depending on the culture conditions.

Germinating seedlings were cultured onto MS medium (Murashige & Skoog, 1962) with B5 vitamins (Gamborg et al 1968), $30 \text{ g} \cdot \text{L}^{-1}$ sucrose and $3 \text{ g} \cdot \text{L}^{-1}$ gelrite in Magenta boxes and incubated in a controlled environment chamber for 30 days with a

16 hour photoperiod under cool white light at $40\text{--}60\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Mature seedlings were subcultured into 125 mL flasks containing 25 mL of basal medium supplemented with zinc at 0, 50, 100, 150 or $200\ \text{mg}\cdot\text{L}^{-1}$ and incubated in the same chamber for 30 days (liquid baslal medium, Table 1).

In experiments designed to determine the zinc uptake in micropropagated *Echinacea* plantlets, petiole explants, 2 cm in length were excised from the 4 week old sterile *Echinacea* plants and subcultured onto induction medium comprising MS media supplemented with thidiazuron (TDZ) ($0.5, 1, 5$ and $10\ \mu\text{mol}\cdot\text{L}^{-1}$) or BAP ($1, 2.5, 5, 7.5, 10, 12.5$ and $15\ \mu\text{mol}\cdot\text{L}^{-1}$) alone or in combination with indoleacetic acid (IAA) at concentrations of 5 and $10\ \mu\text{mol}\cdot\text{L}^{-1}$. Cultures were incubated in a growth cabinet with a 16 hour photoperiod under cool white light at $40\text{--}60\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Regeneration was quantified after 25 and 33 days for all petiole cultures and roots after 33 and 42 days of culture. The resulting regenerants were excised from petioles and subcultured into 125 mL flasks containing 25 mL of basal medium supplemented with zinc at 0, 50, 100, 150 or $200\ \text{mg}\cdot\text{L}^{-1}$ (liquid basal media). The pH of all media was adjusted to 5.7 and experiments were conducted under controlled conditions in a growth chamber with a 16 hour photoperiod at $40\text{--}60\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 30 days.

Plants were harvested on day 45 after treatments were initiated. Plantlets were removed with the shoot and root intact, washed with tap water, rinsed with deionized water and blotted dry with tissue paper. Fresh weight of individual plantlets was determined prior to air drying at 90°C for 48 hours. Each sample was ground with a Waring commercial blender and mineral elements extracted with a closed Teflon vessel using the method of Topper (1990). The zinc content of the samples was determined using an AA55 Varian Atomic Absorption Spectrophotometer or by ICP Analysis. Each experiment consisted of four replicate flasks per treatment and the experiment was repeated twice.

All plants in the control, 50, 100 and 150 mg•L⁻¹ zinc treatment groups appeared normal and healthy. At higher concentrations of zinc in the medium, some inhibition of growth was observed and the rate of plantlet growth was reduced.

The results, shown in Figure 7, demonstrate that zinc accumulation increased with concentration to a maximum of 12475 mg/kg in *Echinacea* plantlets cultured on a medium supplemented with 200 mg •L⁻¹ zinc.

Example 6: Phytofortification of St. John's wort with Lithium

St. John's wort seedlings were obtained from sterilized seeds germinated in a controlled environment. St. John's wort seeds were sterilized by immersing in a 70% ethanol solution for 5 s, followed by an immersion in a 30% solution of 5.4% sodium hypochlorite (Lilo Products, Hamilton, Ontario) in water with one drop of Tween 20 per 500 mL for 20 min, and a three times rinse in sterile distilled water. Sterile seeds were germinated and maintained on water agar (8 g•L⁻¹) for 16 days in darkness in a growth cabinet at 24°C. Individual seedlings were cultured in Magenta boxes on a medium containing MS medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al 1968), 30 g•L⁻¹ sucrose and 3 g•L⁻¹ gellan gum (Gelrite, Schweitzerhall Inc., South Plainfield, NJ, USA). Seedling cultures were incubated in a growth cabinet with a 16 hour photoperiod under cool white light at 40-60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After 30 days, seedlings were excised from the Magenta boxes and subcultured into 125 mL flasks containing 25 mL of a basal medium containing MS medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al 1968) and 30 g•L⁻¹ sucrose. The medium (liquid basal medium) was supplemented with lithium at 0, 50, 100, 150 or 200 mg•L⁻¹ and the cultures were incubated on a rotating platform with a 16 h photoperiod for 30 days at 40-60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

In a second series of experiments, the lithium accumulation of regenerated St.

John's wort shoots was determined. Plantlets were regenerated as described previously. Briefly, St. John's wort seeds were surface sterilized as described above and germinated and maintained on water agar ($8 \text{ g} \cdot \text{L}^{-1}$) for 16 days in darkness in a growth cabinet at 24°C . Hypocotyl sections were excised from sterile etiolated seedlings and cultured on a medium containing MS medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al 1968), $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, $5 \mu\text{mol} \cdot \text{L}^{-1}$ of the cytokinin thidiazuron (pH was adjusted to 5.7 and $3 \text{ g} \cdot \text{L}^{-1}$ gellan gum (Gelrite, Schweitzerhall Inc., South Plainfield, NJ, USA) was added to the medium prior to autoclaving). After 9 days, the hypocotyl sections were subcultured on the same medium devoid of plant growth regulators (thidiazuron) for the development of regenerants.

All cultures were incubated in a growth cabinet with a 16 hour photoperiod under cool white light at $40\text{-}60 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. After 30 days of culture, explants with developing *de novo* shoots and sterile cultured seedlings were transferred to 125 mL flasks containing 25 mL of a basal medium containing MS medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al 1968) and $30 \text{ g} \cdot \text{L}^{-1}$ sucrose. The medium (liquid basal medium) was supplemented with lithium at 0, 50, 100, 150 or $200 \text{ mg} \cdot \text{L}^{-1}$ lithium and the cultures were incubated on a rotating platform with a 16 h photoperiod for 30 days at $40\text{-}60 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Each experiment consisted of four replicate flasks per treatment and the experiment was repeated twice.

Samples were harvested from the cultures analysed for lithium with the same protocol as described above. St. John's wort plantlets at all treatment levels appeared healthy and growth rate was unaffected by lithium supplementation of the culture medium.

The results, shown in Figure 8, demonstrate that lithium accumulated to a concentration of approximately 2000 mg/kg in St. John's wort plantlets cultured on a

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

All publications, patents and patent applications are herein incorporated by reference.

[illegible]

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Consumer Safety Symposium on Dietary Supplements and Herbs (1998) New Good Housekeeping Institute study finds drastic discrepancy in potencies of popular herbal supplement. Good Housekeeping Institute. New York, NY. March 3, 1998.

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ARTICLE 34 AMO'T

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for the *in vitro* micropropagation and phytofortification of a phytopharmaceutical plant comprising:
 - a) culturing a sterile explant of said phytopharmaceutical plant on an induction medium comprising at least one plant growth regulator having cytokinin activity, to form regenerated tissue;
 - b) transferring said regenerated tissue to a basal medium and culturing to form plantlets; and
 - c) subculturing said plantlets onto a basal medium containing at least one additive of interest, to allow uptake and accumulation of said at least one additive of interest in a bio-available form in said plantlet.
2. The method of claim 1, wherein after said step of culturing (step a)), and prior to said step of transferring (step c)), said regenerated tissue is placed on a basal medium and subcultured to allow optimized formation of regenerated tissue; and
3. The method of claim 1 wherein after said step of transferring (step b)), said plantlet is transferred to a hydroponic environment with a recycling solution containing at least one additive of interest to allow uptake and accumulation of said at least one additive of interest in a bioavailable form within said plantlet or seedling.
4. The method according to any one of claim 1, 2 or 3, wherein in said culturing step, said at least one additive of interest is selected from boron, calcium, chloride, chromium, cobalt, copper, iron, lithium, iodine, magnesium, manganese, molybdenum, nickel, phosphorous, potassium, selenium, silicon, sodium, sulphur, tin, vanadium and zinc.
5. The method of any one of claims 1 to 4, wherein said phytopharmaceutical plant is selected from the group consisting of:
 - Achillea millefolium
 - Achyranthes bidentata

ARTICLE 34 AND 'T

Aconitum napellus
Adonis aestivalis
Agastache mexicana
Agrimonia eupatoria
Agathosma betulina
Allium sp
Anchusa officinalis
Anemopsis californica
Angelica dahurica
Angelica polymorpha sinensis (A. sinensis)
Arnica Montana
Ammi visnaga
Arctostaphylos uva-ursi
Asclepias tuberosa
Astragalus membranaceus
Astragalus chinensis
Baphicacanthus cusia
Bixa orellana
Bupleurum falcatum
Brugmansia (Datura) spp.
Campanula rapunculus
Carum roxburgianum
Carum copticum
Cassia tora
Chamaelirium luteum
Chimaphila umbellata
Commiphora africana
Conium maculatum
Crithium maritimum
Datura metel (Datura alba)
Datura inoxia
Dracocephalum moldavica
Echinacea sp.

ARTICLE 34 HINDI

Eclipta alba (E. prostrata)
Ephedra nevadensis
Eriodictyon californicum
Eucommia ulmoides
Eupatorium perfoliatum
Filipendula vulgaris (F. hexapetala)
Gaultheria procumbens
Geum urbanum
Houttuynia cordata
Hydrocotyle asiatica (Centella asiatica)
Hypericum perforatum cv. Anthos
Inula helenium
Jatropha curcas
Leptospermum scoparium
Lespedeza capitata
Ligusticum porteri
Ligustrum lucidum
Lithospermum officinale
Lycium barbarum
Mucuna pruriens
Mandragora officinarum
Origanum dictamnus
Parietaria judaica (P. officinalis)
Phyllanthus emblica
Picrasma excelsa
Piniella ternate
Pogostemon patchouli
Polygonum multiflorum
Porophyllum ruderale ssp. macrocephalum
Prunella vulgaris
Pueraria lobata (P. thunbergiana)
Rauvolfia serpentina
Rivea corymbosa

ARTICLE 34 Amort

Sanguinaria Canadensis
Satureja douglasii
Schizonepeta tenuifolia
Scutellaria baicalensis
Solanum xanthocarpum (S. surattense)
Sutherlandia frutescens
Tabebuia impetiginosa
Tanacetum parthenium
Tribulus terrestris
Trichosanthes kirilowii
Turnera diffusa
Voacanga africana, and
Withania somnifera

6. The method according to claim 5, wherein said phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea* sp. and feverfew (*Tanacetum parthenium*).

7. The method according to any one of claims 1 to 6, wherein said at one plant growth regulator having cytokinin activity is selected from the group consisting of thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N(-phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma,gamma-dimethylallylamino purine).

8. The method according to claim 7, wherein said at least one plant growth regulator having cytokinin activity is selected from thidiazuron (TDZ) and benzylaminopurine (BAP).

9. The method according to claim 8, wherein said induction medium comprises from about 0.001 to about 25 $\mu\text{mol}\cdot\text{L}^{-1}$ of said at least plant growth regulator having cytokinin activity.

10. The method according to claim 8, wherein said sterile explant is maintained on said induction medium from about 1 to about 50 days.

11. The method according to any one of claims 1 to 10, wherein said explant is selected from the seed, petiole, hypocotyl, stem, cotyledon and leaf.
12. The method according to any one of claims 1 to 4, wherein said phytopharmaceutical plant is St. John's wort.
13. The method according to claim 12, wherein said plant growth regulator having cytokinin activity is thidiazuron.
14. The method according to claim 13, wherein the induction medium comprises thiadiazuron from about 0.001 to about 25 $\mu\text{mol}\cdot\text{L}^{-1}$.
15. The method according to claim 14, wherein the induction medium comprises thiadiazuron from about 4 to about 10 $\mu\text{mol}\cdot\text{L}^{-1}$.
16. The method according to claim 12, wherein said sterile explant is maintained on said induction medium from about 1 to about 15 days.
17. The method according to claim 16, wherein said sterile explant is maintained on said induction medium from about 8 to about 10 days.
18. The method according to claim 12, wherein said explant is etiolated hypocotyl.
19. The method according to any one of claims 1 to 4, wherein the phytopharmaceutical plant is *Echinacea sp.*
20. The method according to claim 19, wherein said plant growth regulator having cytokinin activity is selected from the group consisting of thidiazuron and benzylaminopurine.
21. The method according to claim 20, wherein said induction medium comprises from about 0.001 to about 25 $\mu\text{mol}\cdot\text{L}^{-1}$ of said plant growth regulator having cytokinin activity.

22. The method according to claim 20, wherein said plant growth regulator having cytokinin activity is from about 1.0 to about 15 $\mu\text{mol}\cdot\text{L}^{-1}$.
23. The method according to claim 19, wherein said sterile explant is maintained on said induction medium from about 1 to about 50 days.
24. The method according to claim 23, wherein said sterile explant is maintained on said induction medium from about 10 to about 35 days.
25. The method according to claim 19, wherein said explant is petiole.
26. The method according to any one of claims 1 to 4, wherein said phytopharmaceutical plant is Huang qin.
27. The method according to claim 26, wherein said plant growth regulator having cytokinin activity is thidiazuron.
28. The method according to claim 27, wherein said induction medium comprises from about 0.001 to about 25 $\mu\text{mol}\cdot\text{L}^{-1}$ of said plant growth regulator having cytokinin activity.
29. The method according to claim 28, wherein said plant growth regulator having cytokinin activity is from about 1.5 to about 20 $\mu\text{mol}\cdot\text{L}^{-1}$.
30. The method according to claim 26, wherein said sterile explant is maintained on said induction medium from about 1 to about 30 days.
31. The method according to claim 30, wherein said sterile explant is maintained on said induction medium from about 14 to about 20 days.
32. The method according to claim 26, wherein said explant is selected from seeds, hypocotyl and stems.

ARTICLE 34 AND IT

33. The method according to any one of claims 1 to 4, wherein the phytopharmaceutical plant is feverfew.
34. The method according to claim 33 wherein said plant growth regulator having cytokinin activity is thidiazuron.
35. The method according to claim 34, wherein said induction medium comprises from about 0.001 to about 25 $\mu\text{mol}\cdot\text{L}^{-1}$ of said plant growth regulator having cytokinin activity.
36. The method according to claim 35, wherein said plant growth regulator having cytokinin activity is from about 2.0 to about 8.0 $\mu\text{mol}\cdot\text{L}^{-1}$
37. The method according to claim 33, wherein said sterile explant is maintained on said induction medium from about 1 to about 50 days.
38. The method according to claim 37, wherein said sterile explant is maintained on said induction medium from about 20 to about 35 days.
39. The method according to claim 33, wherein the explant is selected from leaf, stem, petiole and hypocotyl.
40. The method according to claim 4, wherein said at least one additive of interest is zinc.
41. A method according to claim 4, wherein said at least one additive of interest is lithium.
42. The method according to claim 4, wherein said at least one additive of interest within said basal medium, is from about 0.001 to about 500 $\text{mg}\cdot\text{L}^{-1}$.
44. The method according to claim 2, wherein, in said transferring step, said regenerated tissue is subcultured for about 1 to about 15 days.

ARTICLE 34 Am'd.

45 A method for phytofortification of an *in vitro*-grown phytopharmaceutical plant comprising:

- a) culturing a sterile seedling, explant or regenerated tissues to form a plantlet; and
- b) subculturing said plantlet onto a basal medium containing at least one additive of interest, to allow uptake and accumulation of said at least one additive of interest in a bio-available form in said plantlet.

46. The method according to claim 45, wherein, in said step of culturing, said plantlets are produced either:

- a) on a sterile explant of said phytopharmaceutical plant grown on an induction medium comprising at least one plant growth regulator having cytokinin activity, or
- b) grown from a sterile seed, or
- c) seedling in culture.

47. The method according to claim 46, wherein said at one plant growth regulator having cytokinin activity is selected from the group consisting of thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N(-phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma,gamma-dimethylallylamino purine).

48. A phytopharmaceutical plant prepared by the method of any one claims 1 to 4, or 45 to 47 and comprising an elevated level of said additive of interest when compared to a plant grown in the absence of said additive of interest.

49. A method for the *in vitro* micropropagation involving *de novo* shoot formation of non-meristematic tissue of a phytopharmaceutical plant comprising:

- a) culturing a sterile explant of said phytopharmaceutical plant on an induction medium comprising one or more plant growth regulators having cytokinin activity, to form regenerated tissue; and
- b) transferring said regenerated tissue to a basal medium and culturing to form plantlets.

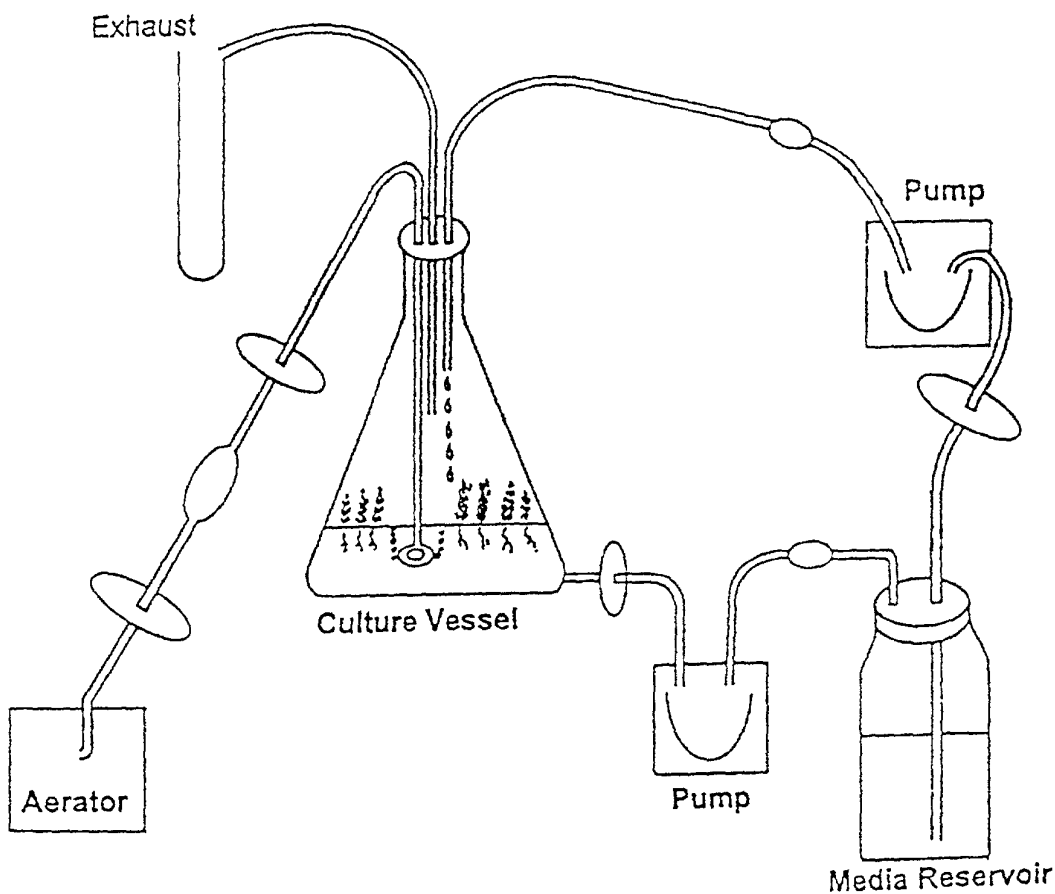


FIGURE 1

WO 00/57690

Inventor: SAXENA et al.
Docket No : 270 60USWO
Title. MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL
PLANTS
Attorney Name: Gregory A. Sebold
Phone No.. 612.336 4728
Sheet 2 of 12

/CA00/00305



FIGURE 2A

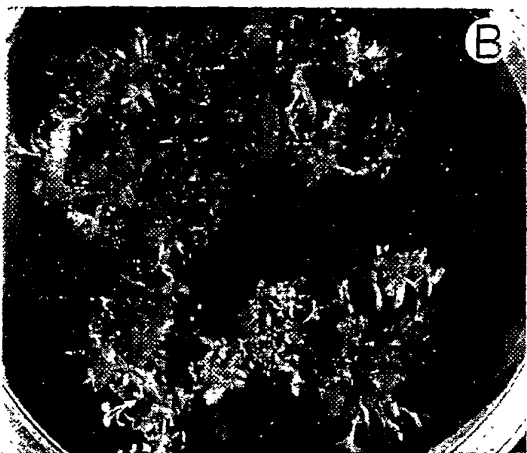


FIGURE 2B



FIGURE 2C

A

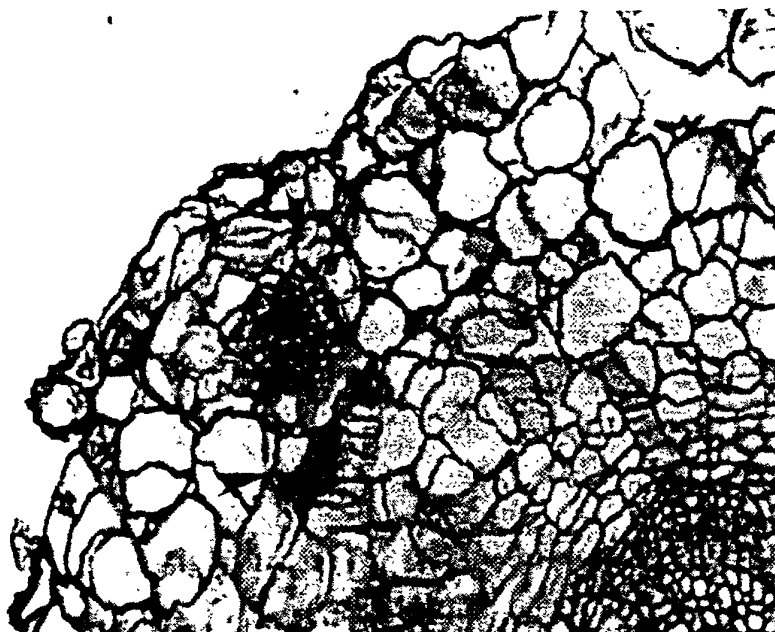


FIGURE 3A

B

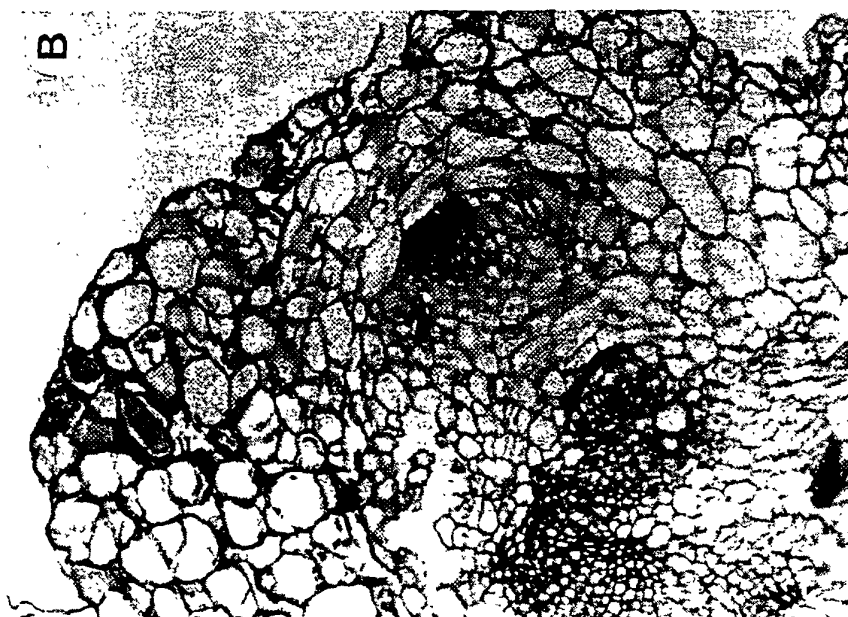


FIGURE 3B

WO 00/57690

Inventor: SAXENA et al.

Docket No. 270.60USWO

Title: MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL PLANTS

Attorney Name: Gregory A. Sebold

Phone No.: 612.336.4728

Sheet 4 of 12

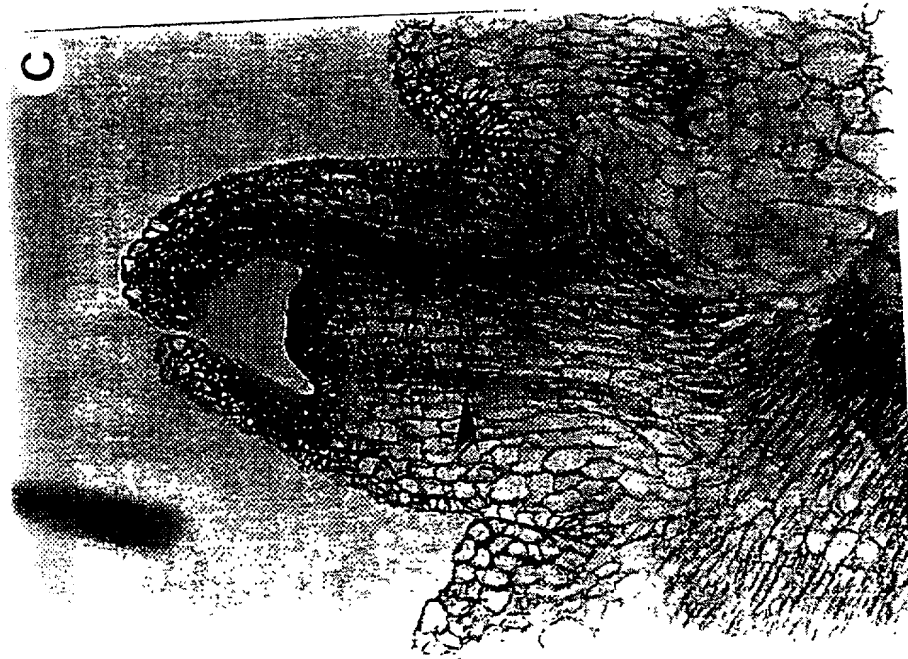


FIGURE 3C

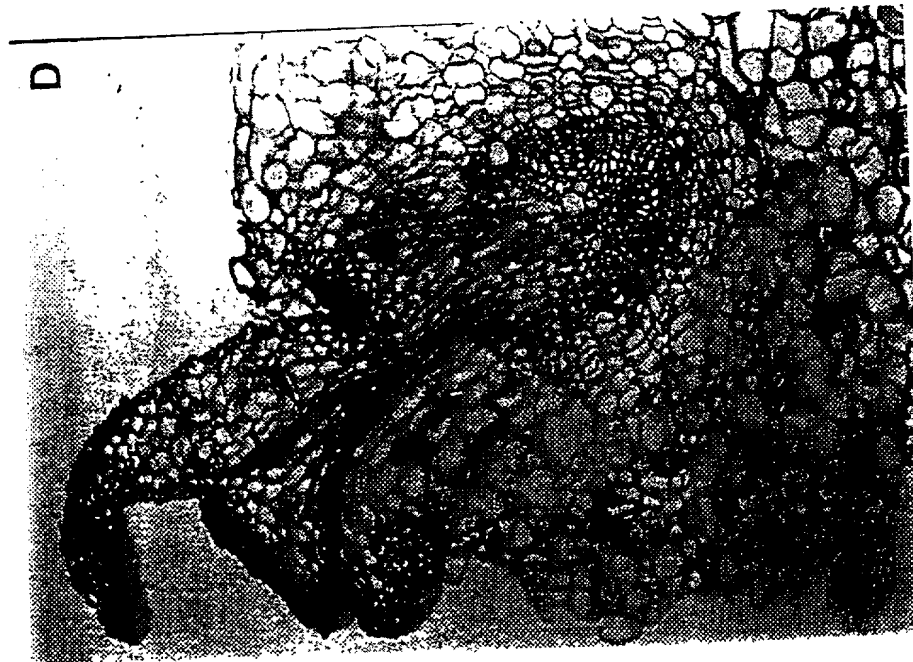


FIGURE 3D

WO 00/57690

Inventor: SAXENA et al.
Docket No : 270.60USWO
Title. MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL
PLANTS
Attorney Name: Gregory A. Sebald
Phone No.. 612.336.4728
Sheet 5 of 12

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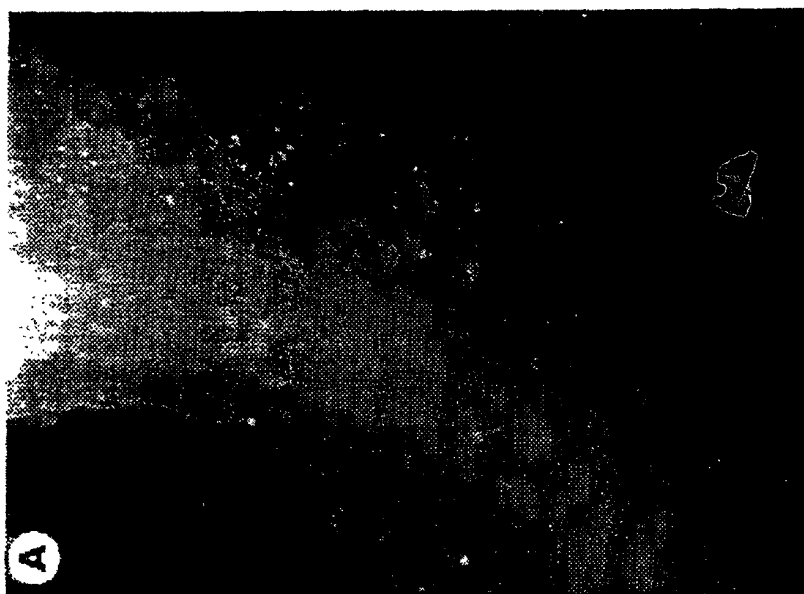


FIGURE 4A



FIGURE 4B

WO 00/57690

Inventor: SAXENA et al.
Docket No.: 270.60USWO
Title: MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL PLANTS
Attorney Name: Gregory A. Sebald
Phone No : 612.336.4728
Sheet 6 of 12

/CA00/00305



FIGURE 4C



FIGURE 4D

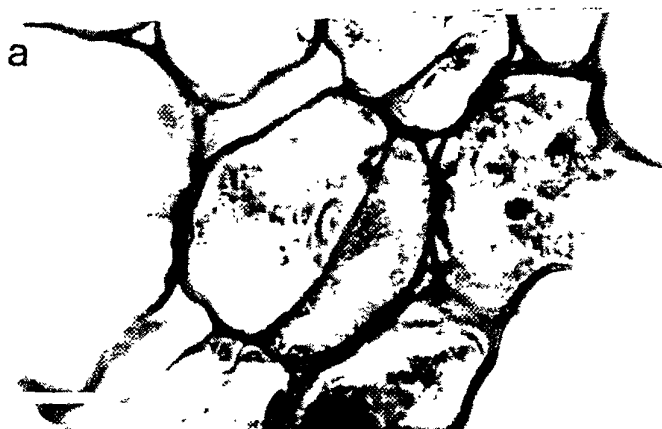


FIGURE 5A



FIGURE 5B

WO 00/57690

Inventor: SAXENA et al
Docket No.: 270.60USWO
Title: MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL
PLANTS
Attorney Name: Gregory A. Sebold
Phone No.: 612 336 4728
Sheet 8 of 12

T/CA00/00305

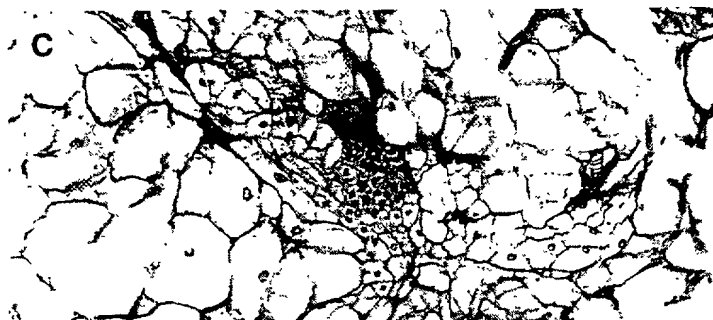


FIGURE 5C

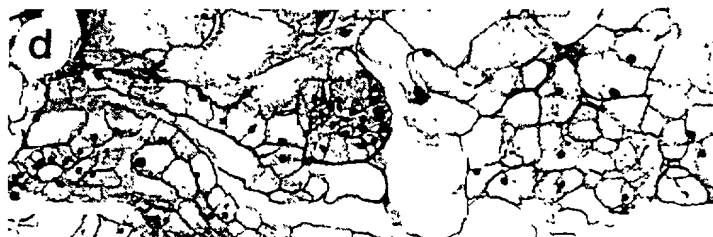


FIGURE 5D



FIGURE 5E

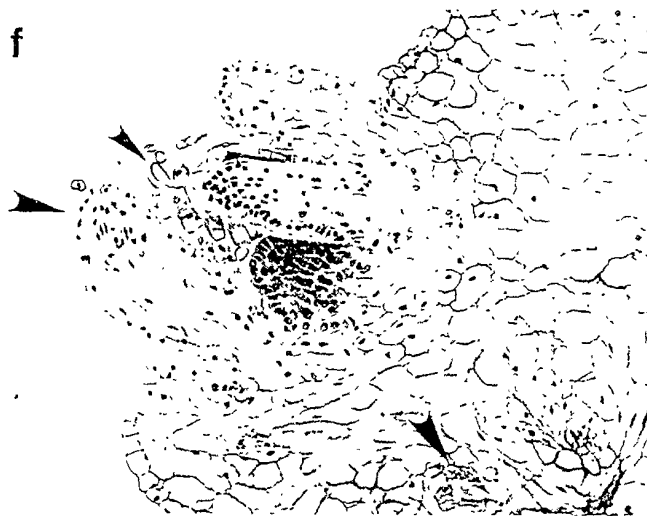


FIGURE 5F

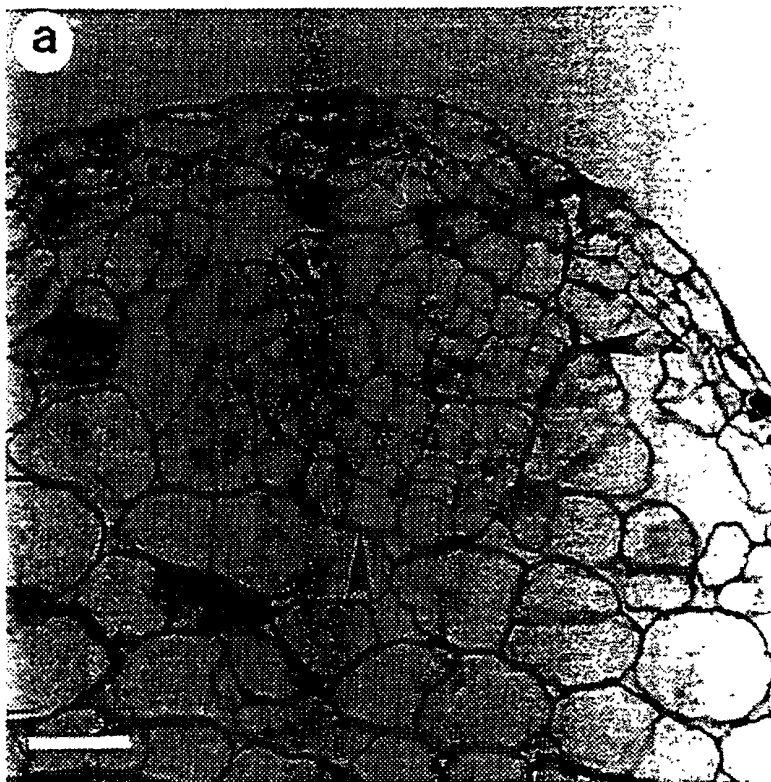


FIGURE 6A

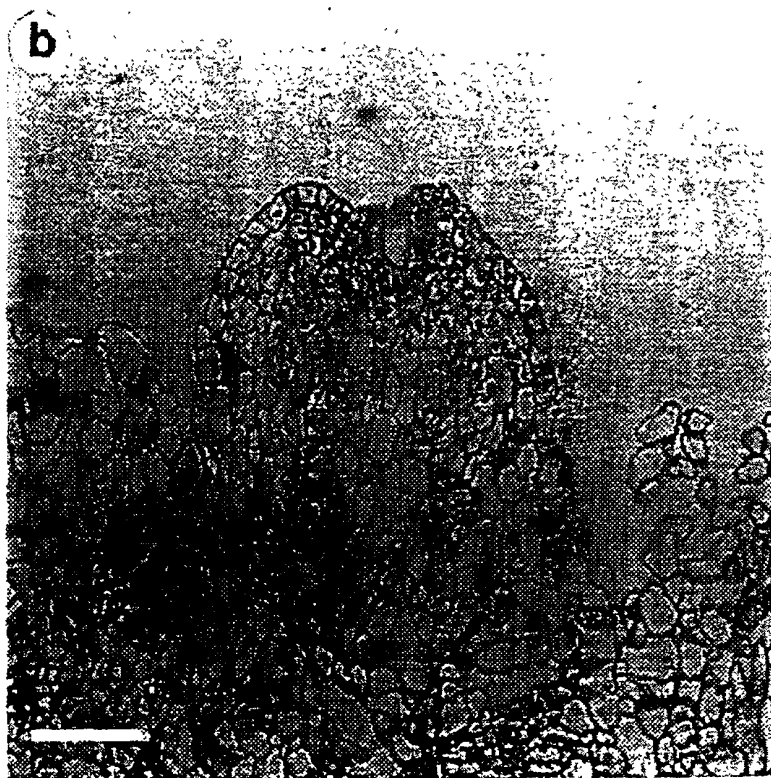


FIGURE 6B

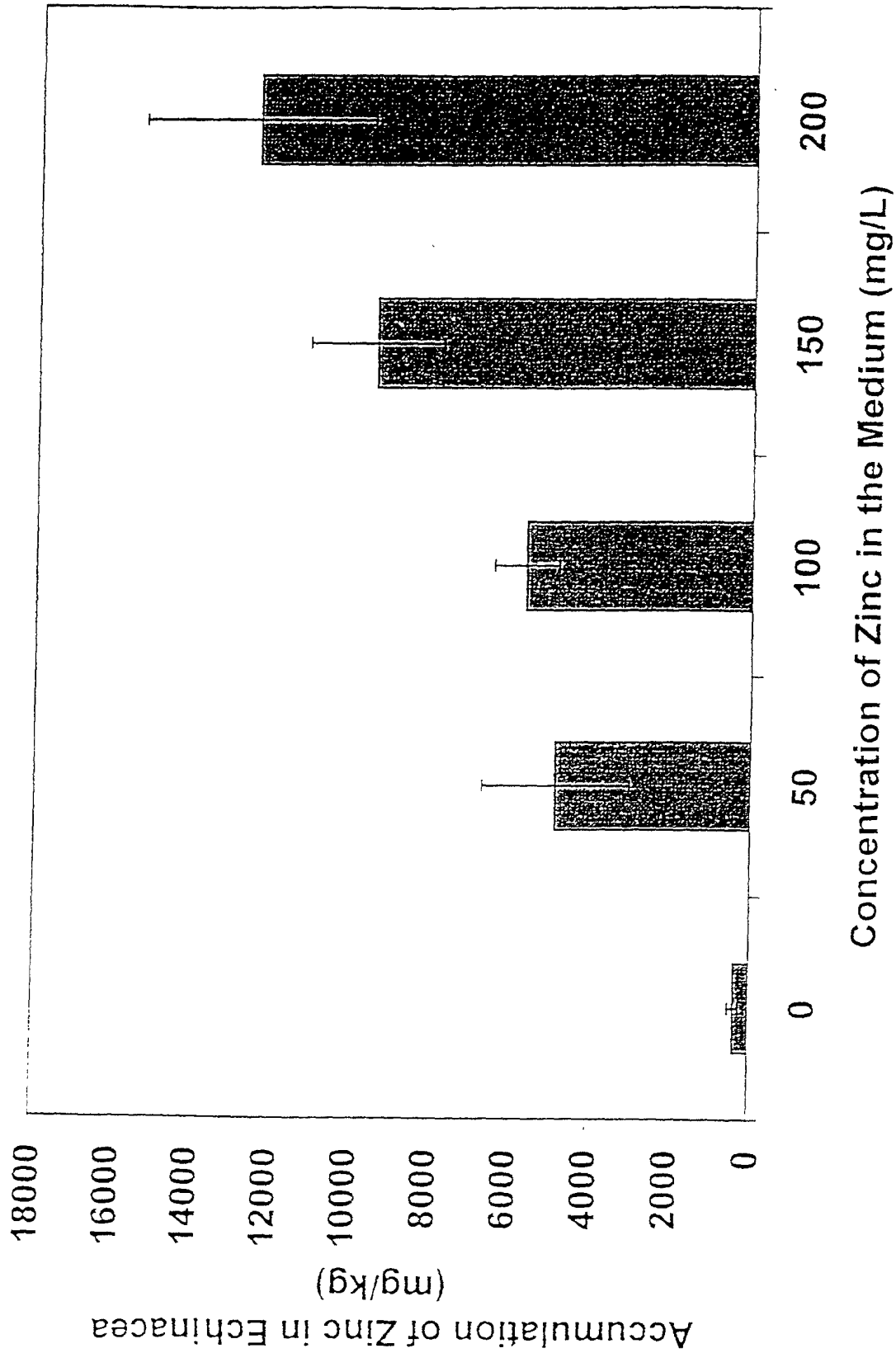


FIGURE 7

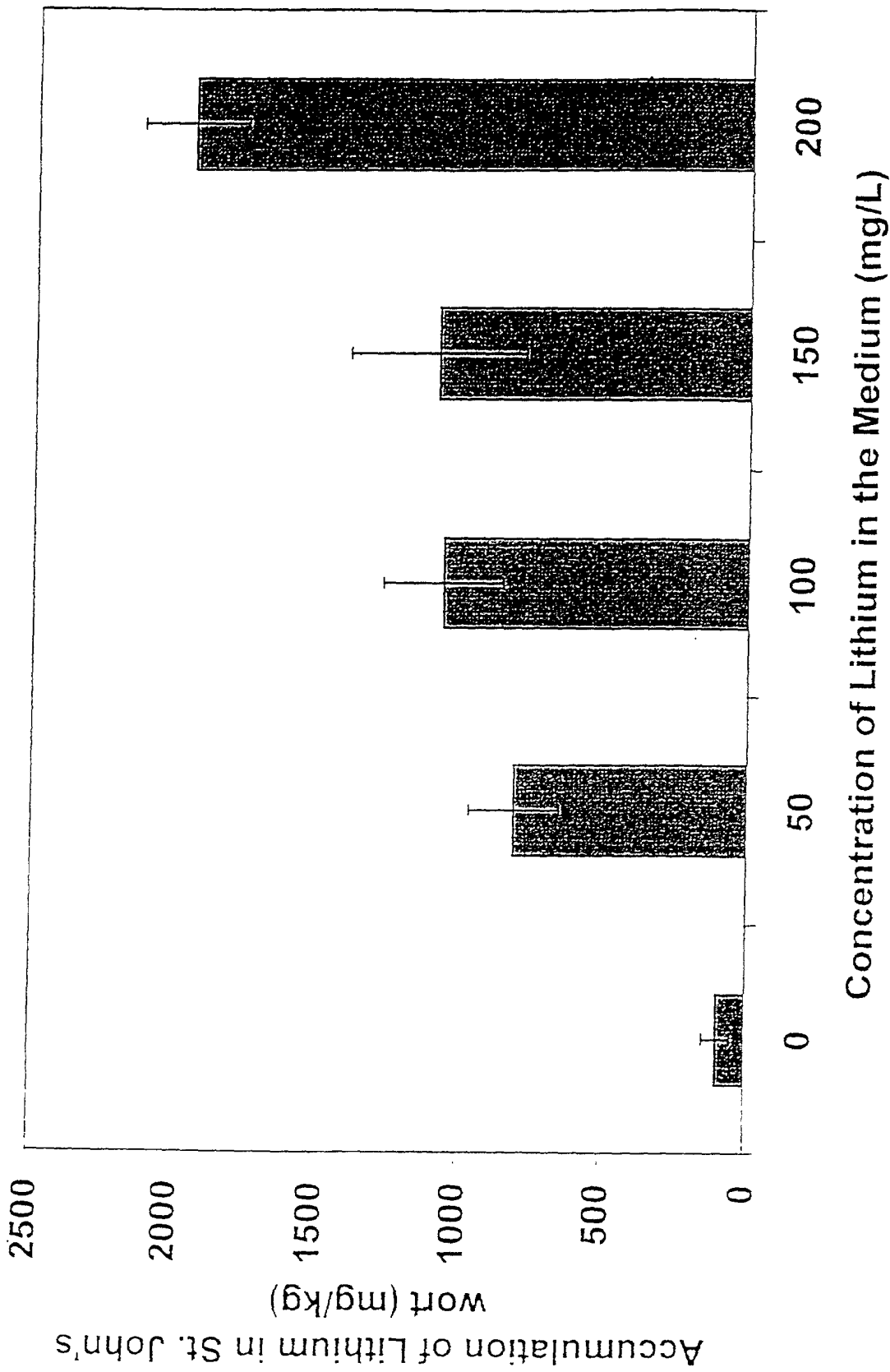


FIGURE 8

MERCHANT & GOULD P.C.

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:
MICROPROPAGATION AND PRODUCTION OF PHYTOPHARMACEUTICAL PLANTS

The specification of which

a. ☐ is attached hereto

b. ☒ was filed on as application serial no. and was amended on (if applicable) (in the case of a PCT-filed application) described and claimed in international no. PCT/CA00/00305 filed March 24, 2000 and as amended on March 19, 2001 (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

a. ☐ no such applications have been filed.

b. ☒ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
Canada	2,267,012	25 March 1999	
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)
60/151,045	27 August 1999

I acknowledge the duty to disclose information that is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (reprinted below):

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim;

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

(e) In any continuation-in-part application, the duty under this section includes the duty to disclose to the Office all information known to the person to be material to patentability, as defined in paragraph (b) of this section, which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

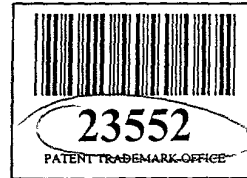
Albrecht, John W.	<u>Reg. No. 40,481</u>	Larson, James A.	<u>Reg. No. 40,443</u>
Ali, M. Jeffer	<u>Reg. No. 46,359</u>	Leonard, Christopher J.	<u>Reg. No. 41,940</u>
Altera, Allan G.	<u>Reg. No. 40,274</u>	Liepa, Mara E.	<u>Reg. No. 40,066</u>
Anderson, Gregg I.	<u>Reg. No. 28,828</u>	Lindquist, Timothy A.	<u>Reg. No. 40,701</u>
Batzli, Brian H.	<u>Reg. No. 32,960</u>	Lown, Jean A.	<u>Reg. No. P-48,428</u>
Beard, John L.	<u>Reg. No. 27,612</u>	Mayfield, Denise L.	<u>Reg. No. 33,732</u>
Berns, John M.	<u>Reg. No. 43,496</u>	McDonald, Daniel W.	<u>Reg. No. 32,044</u>
Branch, John W.	<u>Reg. No. 41,633</u>	McIntyre, Jr., William F.	<u>Reg. No. 44,921</u>
Bremer, Dennis C.	<u>Reg. No. 40,528</u>	Mitchem, M. Todd	<u>Reg. No. 40,731</u>
Brown, Jeffrey C.	<u>Reg. No. 41,643</u>	Mueller, Douglas P.	<u>Reg. No. 30,300</u>
Bruess, Steven C.	<u>Reg. No. 34,130</u>	Nelson, Anna M.	<u>Reg. No. P-48,935</u>
Byrne, Linda M.	<u>Reg. No. 32,404</u>	Parsons, Nancy J.	<u>Reg. No. 40,364</u>
Campbell, Keith	<u>Reg. No. 46,597</u>	Pauly, Daniel M.	<u>Reg. No. 40,123</u>
Carlson, Alan G.	<u>Reg. No. 25,959</u>	Phillips, John B.	<u>Reg. No. 37,206</u>
Caspers, Philip P.	<u>Reg. No. 33,227</u>	Pino, Mark J.	<u>Reg. No. 43,858</u>
Clifford, John A.	<u>Reg. No. 30,247</u>	Prendergast, Paul	<u>Reg. No. 46,068</u>
Cook, Jeffrey	<u>Reg. No. P-48,649</u>	Pytel, Melissa J.	<u>Reg. No. 41,512</u>
Daignault, Ronald A.	<u>Reg. No. 25,968</u>	Qualey, Terry	<u>Reg. No. 25,148</u>
Daley, Dennis R.	<u>Reg. No. 34,994</u>	Reich, John C.	<u>Reg. No. 37,703</u>
Dalglish, Leslie E.	<u>Reg. No. 40,579</u>	Reiland, Earl D.	<u>Reg. No. 25,767</u>
Daulton, Julie R.	<u>Reg. No. 36,414</u>	Samuels, Lisa A.	<u>Reg. No. 43,080</u>
DeVries, Smith, Katherine M.	<u>Reg. No. 42,157</u>	Schmaltz, David G.	<u>Reg. No. 39,828</u>
DiPietro, Mark J.	<u>Reg. No. 28,707</u>	Schuman, Mark D.	<u>Reg. No. 31,197</u>
Doscorch, Matthew A.	<u>Reg. No. P-48,957</u>	Schumann, Michael D.	<u>Reg. No. 30,422</u>
Edell, Robert T.	<u>Reg. No. 20,187</u>	Scull, Timothy B.	<u>Reg. No. 42,137</u>
Epp Ryan, Sandra	<u>Reg. No. 39,667</u>	Sebald, Gregory A.	<u>Reg. No. 33,280</u>
Glance, Robert J.	<u>Reg. No. 40,620</u>	Skoog, Mark T.	<u>Reg. No. 40,178</u>
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Gould, John D.	<u>Reg. No. 18,223</u>	Sullivan, Timothy	<u>Reg. No. 47,981</u>
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Gresens, John J.	<u>Reg. No. 33,112</u>	Swenson, Erik G.	<u>Reg. No. 45,147</u>
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Hamre, Curtis B.	<u>Reg. No. 29,165</u>	Trembath, Jon R.	<u>Reg. No. 38,344</u>
Harrison, Kevin C.	<u>Reg. No. 46,759</u>	Tunheim, Marcia A.	<u>Reg. No. 42,189</u>
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Hope, Leonard J.	<u>Reg. No. 44,774</u>	Weaver, Paul L.	<u>Reg. No. P-48,640</u>
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Kaseburg, Frederick A.	<u>Reg. No. 47,695</u>	Wier, David D.	<u>Reg. No. P-48,229</u>
Kettelberger, Denise	<u>Reg. No. 33,924</u>	Williams, Douglas J.	<u>Reg. No. 27,054</u>
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Kowalchyk, Alan W.	<u>Reg. No. 31,535</u>	Wu, Tong	<u>Reg. No. 43,361</u>
Kowalchyk, Katherine M.	<u>Reg. No. 36,848</u>	Young, Thomas	<u>Reg. No. 25,796</u>
Lacy, Paul E.	<u>Reg. No. 38,946</u>	Zeuli, Anthony R.	<u>Reg. No. 45,255</u>

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Merchant & Gould P.C. to the contrary.

I understand that the execution of this document, and the grant of a power of attorney, does not in itself establish an attorney-client relationship between the undersigned and the law firm Merchant & Gould P.C., or any of its attorneys.

Please direct all correspondence in this case to Merchant & Gould P.C. at the address indicated below:

Merchant & Gould P.C.
P.O. Box 2903
Minneapolis, MN 55402-0903



hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2 1-00	Full Name Of Inventor	Family Name Saxena	First Given Name Praveen	Second Given Name K.
0	Residence & Citizenship	City Guelph CAX	State or Foreign Country Ontario	Country of Citizenship Canada
1	Mailing Address	Address 32 Fieldstone Road	City Guelph	State & Zip Code/Country Ontario N1L 1B4 / Canada
Signature of Inventor 201: X <i>Praveen Saxena</i>			Date: X 14 Nov 2001	
2 2-00	Full Name Of Inventor	Family Name Murch	First Given Name Susan	Second Given Name J.
0	Residence & Citizenship	City Cambridge CAX	State or Foreign Country Ontario	Country of Citizenship Canada
2	Mailing Address	Address 43 William Street	City Cambridge	State & Zip Code/Country Ontario N3H 3W6 / Canada
Signature of Inventor 202: X <i>Susan Murch</i>			Date: X 10 Nov 01	
3 3-00	Full Name Of Inventor	Family Name Krishnaraj	First Given Name Sankaran	Second Given Name
0	Residence & Citizenship	City Guelph CAX	State or Foreign Country Ontario	Country of Citizenship Canada
3	Mailing Address	Address 128-78 College Avenue West	City Guelph	State & Zip Code/Country Ontario N1G 4S7 / Canada
Signature of Inventor 203: X <i>Sankaran Krishnaraj</i>			Date: X 14 Nov 2001	
2 4-00	Full Name Of Inventor	Family Name Slimmon	First Given Name Tannis	Second Given Name Y.
0	Residence & Citizenship	City Guelph CAX	State or Foreign Country Ontario	Country of Citizenship Canada
4	Mailing Address	Address 71 Grange Street	City Guelph	State & Zip Code/Country Ontario N1E 2V1 / Canada
Signature of Inventor 204: X <i>Tannis Slimmon</i>			Date: X November 15/01	